İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF HYDROGEN PEROXIDE RESISTANT YEAST

M.Sc. Thesis by
Meliha Merve HIZ

Department : **Advanced Technologies**

Programme : **Molecular Biology–Genetics and Biotechnology**

JANUARY 2010

İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF HYDROGEN PEROXIDE RESISTANT YEAST

M.Sc. Thesis by
Meliha Merve HIZ
(521071043)

Date of submission : 07 December 2009
Date of defence examination: 29 January 2010

Supervisor (Chairman) : Assoc. Prof. Dr. Zeynep Petek ÇAKAR (ITU)
Members of the Examining Committee : Prof. Dr. Tulay TÜLUN (ITU)
Assist. Prof. Dr. Fatma Neşe KÖK (ITU)

OCAK 2010

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE HİDROJEN PEROKSİDE DİRENÇLİ MAYALARIN GELİŞTİRİLMESİ

YÜKSEK LİSANS TEZİ
Meliha Merve HIZ
(521071043)

Tezin Enstitüye Verildiği Tarih : 07 Aralık 2009
Tezin Savunulduğu Tarih : 29 Ocak 2010

Tez Danışmanı : Doç. Dr. Zeynep Petek ÇAKAR (İTÜ)

Diğer Jüri Üyeleri : Prof. Dr. Tülay TÜLUN (İTÜ)
Yard. Doç. Dr. Fatma Neşe KÖK (İTÜ)

OCAK 2010

FOREWORD

I would like to express my deep gratitude to my supervisor Assoc. Prof. Dr. Zeynep Petek ÇAKAR for the inspiring guidance, support and the opportunities she provided me. It was a great pleasure for me to carry on this thesis under her supervision.

I appreciate Yusuf Sürmeli for sharing for his collaboration during the experiments. He helped for managing the time problems that were encountered in enzyme activity study.I would like to thank Burcu Turanlı, Bahtiyar Yılmaz and Tuğba Aloğlu for sharing their experience with me through the study.

I would like to thank Levent Üge for his effort on technical help for fixing machine and providing material during the study what I needed anytime without any complaint and for sharing his experiences.

I am grateful to Derya Canbaz for her infinite morale support and friendship. She was ready to help in any subjects whenever I requested. She is perfect friend and she has never disappoint me any time. She was always with me, and providing me such strong support throughout that frienship.

I would also thank to especially Berrak Gülçin Balaban and Onur Ercan for their moral, motivation and friendship during my thesis. Berrak has strong character and reliable personality. I would like to thank her for being every time with me in the lab and making the time pass easily.

I would also like to thank to the authorities of The Scientific and Technological Research Council of Turkey "TUBITAK" (2210 programme) and gratefully acknowledge the financial support they provided me for my graduate studies.

Lastly, I am especially grateful to my family; Erol and Nurşen Hız. Thanks for always being there for me during all my life, your love and support meant the world to me. I am greatful to Hale Çaloğlu for her moral incentive in my hard times. I would like to thank my wonderful family for their patience and having every confidence in my ability to succeed, not only during this study but throughout my life. I would not imagine living without their endless love, encouragement and support.

January 2010

Meliha Merve HIZ

Molecular Biologist

TABLE OF CONTENTS

Page

ABBREVIATIONS

µg : Microgram
µl : Microliter
µM : Micromolar
µm : Micrometer
ADP : Adenosine diphosphate
ATP : Adenosine triphosphate
bp : Base pair
BSA : Bovine Serum Albumine
cDNA : Complementary DNA
Da : Dalton
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide
E.coli : *Escherichia coli*
EtBr : Ethidium bromide
EMS : Ethyl Methane Sulphonate
H_2O_2 : Hydrogen Peroxide
GDP : Guanosine di-phosphate
GTP : Guanosine tri-phosphate
Kb : Kilo base
M : Molar
mA : Milliampere
ml : Milliliter
mM : Millimolar
mm : Millimeter
mRNA : Messenger ribonucleic acid
NAD : Nicotinamide adenine dinucleotide (oxidised)
NADH : Nicotinamide adenine dinucleotide (reduced)
NCBI : National Center for Biotechnology Information
nt : Nucleotide
OD : Optical Density
PCR : Polymerase chain reaction
RNA : Ribonucleic acid
pH : Power of hydrogen
ROS : Reactive Oxygen Species
SOD : Superoxide Dismutase
GSH : Glutathione
GRX : Glutaredoxin
rpm : Revolutions per minute
sec : Second
TBE : Tris-borate-EDTA
T_m : Melting temperature
UV : Ultraviolet

LIST OF TABLES

LIST OF FIGURES

EVOLUTIONARY ENGINEERING OF HYDROGEN PEROXIDE RESISTANT YEAST

SUMMARY

Saccharomyces cerevisiae is an aerobic organism, and is constantly exposed to reactive oxygen species (ROS), generated as metabolic byproducts, by respiration. Besides cellular metabolism, reactive oxygen species are also produced by external oxidants. ROS are highly damaging towards cellular constituents, including DNA, lipids and proteins, thus yeast cells have to maintain cellular integrity against reactive oxidants. *S. cerevisiae* is a eukaryotic model organism to provide considerable information on the cellular response against oxidative stress and the defense functions involved in stress response.

In the first part of the study, the aim was to obtain hydrogen peroxide resistant *S. cerevisiae* cells by using an inverse metabolic engineering strategy; evolutionary engineering. For that purpose, pulse selection strategy was applied and yeast cells were exposed to increasing concentrations of H_2O_2. The individual mutants from the final mutant generation were selected randomly and tested for potential cross resistances to other stress types by a high throughput most probable number based technique.

In the second part of the study, the aim was to determine specific catalase activity of the mutant yeasts and the genetic expression profile of selected oxidative stress related genes “YAP1, CTT1, HSP104, AFT1, GLR1and YAP5, in the absence and presence of H_2O_2.

The ultimate aim was to understand molecular mechanism of resistance to hydrogen peroxide stress. The results revealed that highly hydrogen peroxide resistant individuals (up to ~ 2000 fold of the wild type level) which were selected from the that mutant population obtained by evolutionary engineering, also gained cross-resistance to iron-stess. Further transcriptomic and proteomic investigations might help to understand resistance mechanisms of the mutant individuals and the correlation between oxidative stress and iron-stress resistance mechanisms.

EVRİMSEL MÜHENDİSLİK YÖNTEMİ İLE HİDROJEN PEROKSİDE DİRENÇLİ MAYALARIN GELİŞTİRİLMESİ

ÖZET

Saccharomyces cerevisiae bir aerobik organizmadır ve solunum tarafından metabolik yan ürünü olarak üretilen reaktif oksijen türlerine (ROS) maruz kalmaktadır. Bununla beraber, hücresel metabolizma yanında, ROS hücre dışı oksidantlar tarafından da üretilmektedir. ROS, DNA, lipit ve proteinleri içeren hücresel bileşenlere ciddi ölçüde zarar vermektedir. Bu yüzden maya hücreleri, reaktif oksidantlara karşı hücre içeriklerini korumak zorundadırlar. *S.cerevisiae,* oksidatif strese karşı ve stres cevabındaki hücresel savunma fonksiyonları üzerinde önemli bilgiler sağlayan ökaryotik bir model organizmadır.

Çalışmanın ilk bölümünde amaç, bir tersine metabolik mühendislik stratejisi olan evrimsel mühendislik ile hidrojen peroksite dirençli *S. cerevisiae* hücreleri elde etmekti. Bu sebeple, ani (pulse) seçim stratejisi uygulandı ve maya hücreleri artan hidrojen peroksit konsantrasyonlarına maruz bırakıldı. Mutant bireyler, son mutant neslinden rastgele seçildi ve yüksek verimli, en muhtemel sayı tabanlı teknik (MPN) ile diğer stres türlerine karşı potansiyel çapraz dirençleri test edildi.

Çalışmanın ikinci aşamasında amaç, mutant mayaların özgül katalaz aktivitelerini ve H_2O_2 varlığında ve yokluğunda, seçilen oksidatif stresle ilişkili genlerin ("YAP1, CTT1, HSP104, AFT1, GLR1 ve YAP5") genetik anlatım profilini belirlemekti.

Çalışmanın temel amacı, hidrojen peroksit stres direncinin moleküler mekanizmasını anlamaktı. Sonuçlar, evrimsel mühendislik ile son mutant popülâsyonundan elde edilerek seçilen bireylerin, yüksek oranda hidrojen perokside dirençli olanlarının (yabanıl tipin seviyesinin 2000 katı) demir stresine de çapraz direnç kazandıklarını da ortaya koymuştur. İleri transkriptomik ve proteomik araştırmalar, mutant bireylerin direnç mekanizmasını ve oksidatif stres ile demir-stres direnç mekanizmaları arasındaki ilişkiyi anlamaya yardımcı olabilir.

1. INTRODUCTION

1.1 *Saccharomyces cerevisiae*: Brief information

The yeast *Saccharomyces cerevisiae* is a unicellular, eucaryotic organism that belongs to the kingdom Fungi, phylum Ascomycota, class Hemiascomycetales, order Saccharomycetales, family Saccharomycetacea and genus Saccharomyces.

Saccharomyces cerevisiae is a eukaryotic fungus with a genome of individual linear chromosomes enclosed in a nucleus and with cytoplasmic organelles such as endoplasmic reticulum, Golgi apparatus, mitochondria, peroxisomes, and a vacuole analogous to a lysosome (Moat, et al, 2002).

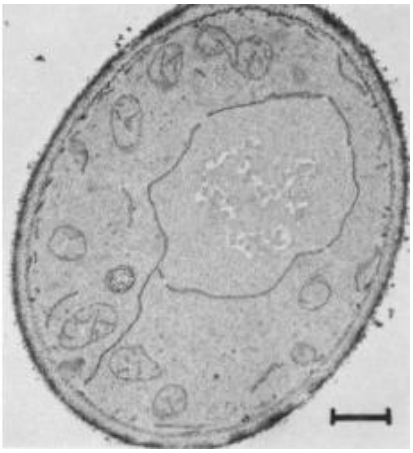


Figure 1. 1 : General view of a section of a cell of *Saccharomyces cerevisiae*. Prominent features include the cell wall, the nucleus with distinct pores in the nuclear membrane, mitochondria with cristae, and inter membranous structures.Bar equals 100 nm.(Moat, et al, 2002)

The predominant form of *Saccharomyces cerevisiae* is unicellular, oval shaped cells and the size of the organism varies between 5–10 μm length and a width of 5–7 μm. It has a tough cell wall and prominent central vacuole. Yeast have a cell wall as the outer layer and the cytoplasmic membrane is between the cell wall and the cell's cytoplasm. The cell wall of yeast are composed of glucan and mannan. The cytoplasmic membrane is composed of proteins and lipids surrounding the yeast and serves as a barrier between the inner cell and its environment (Betsy, et al, 2005).

The cytoplasm of a yeast cell contains cytosol, organelles, and inclusion bodies. Yeast cells have an internal skeleton, the cytoskeleton, that gives the yeast cells shape, its capacity to move, and its ability to arrange its organelles and transport them from one part of the cell to another. The cytoskeleton is composed of a network of protein filaments, The actin filaments and microtubules are two of the most important protein filaments (Lodish, et al, 2001).

Briefly, intermediate filaments provide mechanical strength and resistance to shear stress. Microtubules determine the positions of membrane-enclosed organelles and direct intracellular transport and are involved in cell division in the course of drawing chromosomes to the poles. Microfilaments (composed of actin filaments) determine the shape of the cell's surface and are necessary for whole-cell locomotion (Alberts et al., 2002).

Mitochondria of yeast vary in number and shape. Mitochondirial structure and volume depend on the carbon source of the medium. The mitochondrial genome of *S.cerevisiae* is 80.000 bp in size and encodes eight major proteins for survive. (Leister, et al, 2007). Related to cell activity, mitochondria, peroxisome size, and protein content are able to change. Yeast peroxisomes contain high density proteins and many of these proteins are enzymes that join hydrogen peroxide producing oxidase and catalase and also for β-oxidation parthways. Peroxisomes can also have other different functions depending on the environmental and developmental conditions (Carlile, et al,2001).

S. cerevisiae is able to grow rapidly in rich media under aerobic conditions, a cell population doubling in about 90 min. It has a small genome with 12.057.500 base pairs of DNA that encodes 6000 genes, on 16 chromosomes (Alberts et al, 2002). Because of the importance of yeast as a model system in molecular biology and genetics, the entire genome of *S. cerevisiae* was sequenced and two-thirds of the approximately 6000 identified ORFs have been characterized. The *S. cerevisiae's* genes are compact, having fewer introns and the spaces between the genes are relatively short when compared with complex eukaryotic organisms (Carlile, et al,2001).

*S. cerevisiae'*s growth is "oxidoreductive" under aerobic conditions with critical glucose flux, and "reductive" under anaerobic conditions. (*S. cerevisiae* metabolizes

glucose via the glycolytic (Embden-Meyerhof) pathway to pyruvate. Depending on absence or presence of oxygen, aerobic respiration or anaerobic fermentation occurs. If oxygen is avaliable, pyruvate can be oxidized via the tricarboxylic acid cycle to carbon dioxide and water. In the absence of oxygen, or at high sugar concentrations, alcoholic fermentation occurs, with ethanol and carbon dioxide being produced. (Scheffler, 2008).

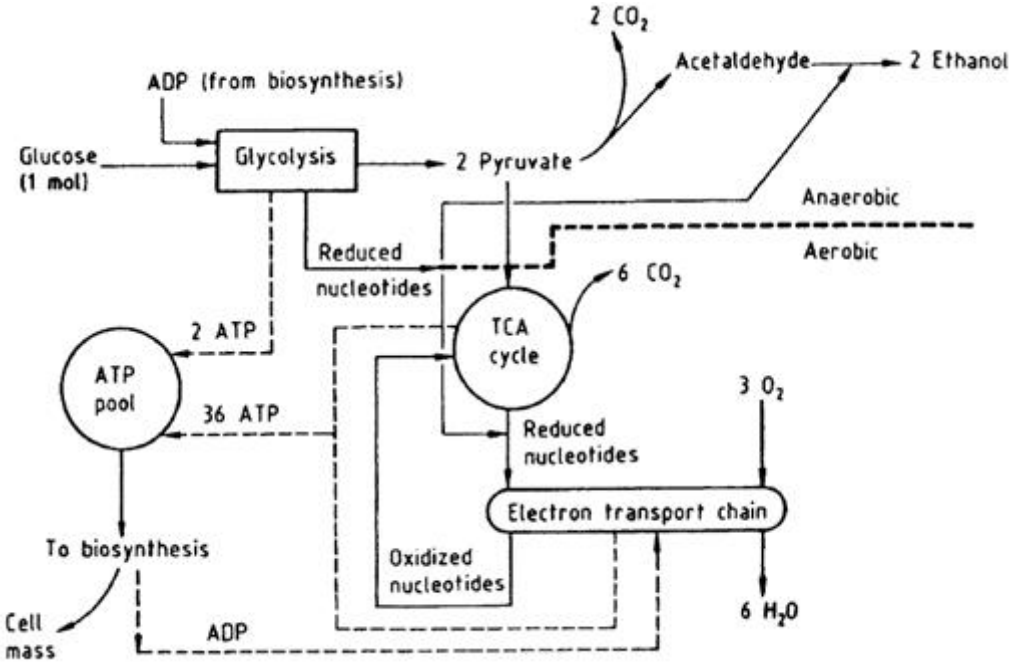


Figure 1.2 : Aerobic and anaerobic catabolism of *Saccharomyces cerevisiae.* *(Roehr, 2001)*

Saccharomyces is a genus of budding yeasts that reproduce asexually by budding or sexually by conjugation, that provides adaptive advantages.When the environment is favorable, rapid asexual reproduction occurs, but in environmental stress conditions, sexual reproduction enhances genetic diversity, and the offspring can adapt to the new environmental conditions easily (Postlethwait, et. al, 2006).

S.cerevisiae reproduces asexually from buds that eventually pinch off to produce new cells. Budding is different from usual cell division. Budding begins with DNA duplication in S phase of interphase. The bud appears on the surface of mother cells. After chromosomes are duplicated, the chromosomes are divided between the bud and mother cell, the bud then enlarges and is separated from the mother cell to form independent cell (Carlile, et. al, 2001).

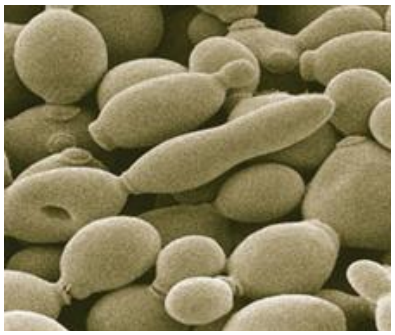


Figure 1.3 : Scanning electron micrograph of budding yeast.(URL-1)

S.cerevisiae reproduces sexually by fusion of two opposite mating types to form diploid cells. Two mating types occur and are designated a and α. The mating is mediated by mating factors. The haploid cells in mating type α produce α factor that are only recognised by a type cell suface receptor and mating type a produces a factor that is only recognised by α type cell suface receptor. The hormones as secreted like mating factor also cause the yeast cells to change in shape, becoming "shmoo"(Figure 1.4).

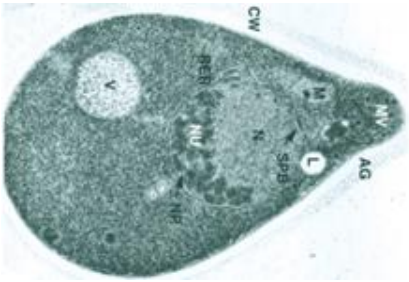


Figure 1.4 : Electron micrograph of a thin section of a shmoo of *Saccharomyces cerevisiae*, fixed freeze-substitution. (Carlile, et. al, 2001)

Cell fusion is followed by nuclear fusion, initiating the diploid phase. At the end of the process, haploid cells of opposite mating type mate to produce a/ α diploids, which carry two copies of each chromosome and diploid form occurs. (Figure 1.5).

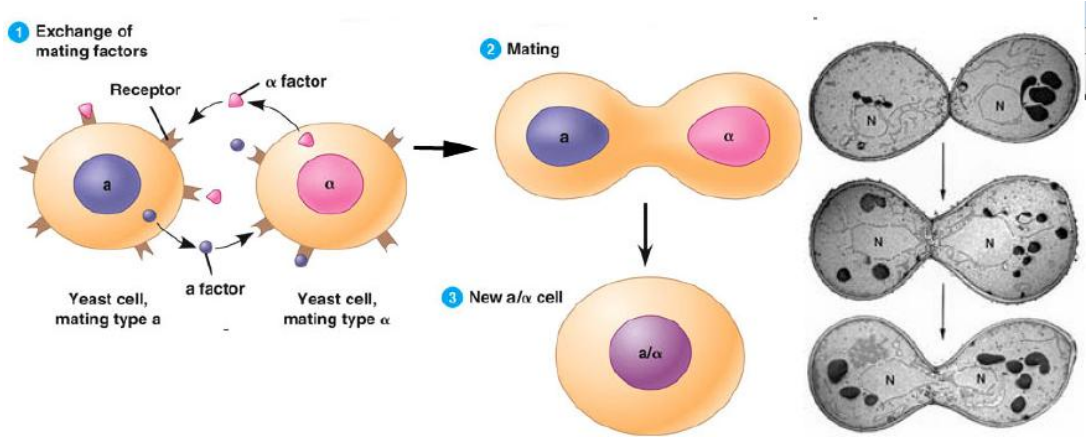


Figure 1.5 : Sexual reproduction of *S.cerevisiae*. (Adopted from URL-1)

Saccharomyces cerevisiae is a well-studied minimal eukaryotic model organism. It is because budding yeast can easily grow in culture with inexpensive media. The basic cellular mechanisms of yeast such as DNA replication, recombination, cell division and metabolism are highly similar to that of higher organisms including humans. Exploration of the genetics of the model organism *S. cerevisiae* has proved useful in numerous ways. Yeast genome is easy and inexpensive to manipulate and mutagenise by using EMS or radiation. By controlling nutrient levels, the researchers can control growth rate and also the switch from diploid to haploid form (Hogg, 2005).

1.2 The importance of *Saccharomyces cerevisiae* in industry

Saccharomyces cerevisiae is used commercially for centuries in manufacturing of bread, beer, and wine. The byproduct of anaerobic fermentation of yeast is ethanol. Alcoholic fermentation by *Saccharomyces* is responsible not only for the production of beer and wine but also, through carbon dioxide formation, for the raising of the dough in bread making. The productivity of yeast in a variety of different processes has improved significantly since genetic methods have been introduced.

Nowadays yeasts are also used to produce pharmaceuticals, vaccines, hormones, blood factors in health-care industries and savoury flavors, enzymes, baking pigments, food acidulants, chemical reductions in food chemical technologies besides fermentation industries (Rehm, et. al, 1991).

The fermentation of sugar to ethanol by yeast has an important place among the different processes that are used in industry. Alcohol fermentation is initiated by adding yeast to a carbon source. *S.cerevisiae* is of major importance for the large scale production of ethanol. *S.cerevisiae* is able to metabolize glucose to ethanol under anaerobic conditions with production of 2 mol each of ethanol, CO_2, and ATP per mol of glucose. *S.cerevisiae* is not able to degrade starch and dextrin, but today, genetically engineered strains have been developed, which are able to utilize lactose, melobiose, xylose, and other materials. Genetically engineered strains of *S. cerevisiae* is useful for the production of alcoholic beverages **(**Roehr, et. al, 2001).

A recombinant strain of *S.cerevisiae* able to express several lactate dehydrogenases (LDHs) accumulated 40% more lactic acid with a yield of 0.44 g of lactic acid per gram of glucose (Skory, et. al, 2003).

A transgenic *S.cerevisiae* is produced Xylitol which is an attractive sweetener used in the food industry. That production in yeast is performed by the expression of xyl1 of *Pichia stipitis*, encoding a xylitose reductase (Govinden, et. al, 2001).

Methyl mercury accumulates under anaerobic conditions and is degraded under aerobic conditions. *Saccharomyces cerevisiae* is able to methylate Hg(II) to methyl mercury under mostly anaerobic conditions.

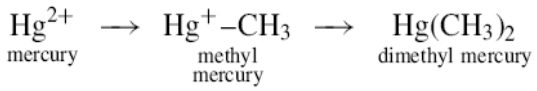


Figure 1.6 : Mercury degragation pathway (Bitton, 2005).

The medical importance and commercial value of *S.cerevisiae* comes from the production of pharmaceuticals. The product of Immunex, Leukine is produced by using *Saccharomyces* expression system. This drug is used against leukemia and lymphoma (Kayser, et. al, 2004).

Saccharomyces cereviseae has been used for the production of a vaccine for hepatitis B. The hepatitis B surface antigen" HBsAg" is produced efficiently in yeast. The HBV infection is successfully performed and immunity is maintained for at least 10 years (Kayser, et. al, 2004).

1.3 Reactive oxygen species and oxidative stress

Yeast cells are unicellular organisms able to grow in both aerobic and anaerobic conditions; but during aerobic growth, incomplete reduction of oxygen to water leads to the formation of redox-active oxygen intermediates.

The complete reduction of one molecule of O_2 to water is a four electron process. Oxidative metabolism continually generates partially reduced species of oxygen, which are more reactive and hence potentially more toxic than O_2 itself. A one-electron reduction of O_2 yields superoxide anion radicals (O_2-), an additional electron yields hydrogen peroxide (H_2O_2) and a third electron yields hydroxyl radicals ($OH^{\cdot}$) and a hydroxide ion (Mathews and Van Holde, 1990).

Not only aerobic metabolism but also β-oxidation of fatty acids and exposure of cells to ionizing radiation, heavy metals and redox-cycling chemicals cause formation of reactive oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\cdot$) (Godon, et al, 1998; Derek, 1998).

The cellular effects of ionizing radiation to damage the cell depend on four-step reactions that start with water radiolysis, split of water into hydroxyl radicals and hydrated electrons, and endproduct of those reactions cause the production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Molin, et al, 2007).

Although oxidants, reactive oxygen species, are produced within the cell; all aerobic organism also produce anti-oxidants and obtain a balance between oxidants and anti-oxidants. When the cells are exposed to excess levels of oxidants, redox equilibrium breaks down and leads to oxidative stres (Scandalios, 1993).

In order to survive, yeast cells induce antioxidant defense mechanisms and adopt new environmental conditions (Costa and Ferreira 2001).

1.4 Oxidative damage on biomolecules

The yeast cells can sense the oxidative stress and develop a stress response to inhibit cellular damage under normal physiological conditions with the help of two different defense systems. The first one is primary defense which neutralizes the reactive oxygen species and the other is secondary defense that repairs molecular damage as well as degrades oxidized molecules.

The conditions that increase production of reactive oxygen species or cause decrease in antioxidant molecules inhibit regulation of defense systems and cause specific cellular damage on proteins, lipids and nucleic acids (Costa and Ferreina 2001).

1.4.1 Oxidative damage on protein

Reactive oxygen species damage proteins directly by oxidation of their amino acid residues and cofactors or indirectly attack via lipid peroxidation.

Protein amino acid residues oxidation cause conformational changes that decrease or prevent activity of proteins when target amino acids are at or close to active site. Hydroxyl radicals which are produced by the Fenton reaction inactivate enzymes

irreversibly, but H_2O_2 inactivates a few enzymes reversibly due to oxidation of thiol groups of cysteine residues at active site (Costa, et al, 2001).

Cofactors are also important and lead to loss of enzyme activity depending on oxidation by reactive oxygen species. Superoxide radicals specifically oxidize 4Fe-4S clusters in enzymes (i.e. mitochondrial aconitase) and release iron from the cluster and inactivate the enzyme (Costa, et al, 2007).

1.4.2 Oxidative damage on nucleic acids

Oxidative stress causes different types of DNA damage such as base lesions, DNA-protein cross-links, single-strand breaks, double strand breaks. Especially base modifications are more important than others due to their lethal or mutagenic effects.

In the case of hydrogen peroxide and superoxide present in *S.cerevisae*, that cause more than usual base oxidation, strand breaks and intrachromosomal recombination occurs. In nucleus, oxidized bases have to be removed and replaced. Rad1p is a nucleotide excision repair protein of damaged DNA in *S.cerevisae* and also two DNA glycosylases Ogg1p and Ntg2p remove damaged bases.

Mitochondrial DNA lacks protective histones thus besides excision repair glycosylase Ntg1p which removes dihydrothymine, urea and rail glycol, the repair enzyme Mgm101p is also present (Costa and Ferreina 2001).

1.4.3 Oxidative damage on lipids

Lipid peroxidation is a process where reactive oxygen species steal electrons from the lipids that are located in cell membranes by an autocatalytic process. Oxidation of polyunsaturated fatty acids causes the production of fatty acid hydro peroxides and their fragmentation to highly reactive lipid products such as epoxides, aldehydes and alkanes (Costa and Ferreina 2001).

The general process of lipid peroxidation consists of three stages: initiation, propagation, and termination (Catala, 2006). The initiation phase of lipid peroxidation includes hydrogen atom abstraction with the help of radicals: hydroxyl ($^{\bullet}OH$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), and possibly $HO_2^{\bullet}$ but not H_2O_2 or $O_2^{-\bullet}$ (Gutteridge, 1998).

During lipid peroxidation, biomolecules such as proteins or amino lipids can be covalently modified by these lipid decomposition products, which damage membrane structure by modifying its physical properties (Catala, 2009).

Although yeast cells are unable to synthesize polyunsaturated fatty acids, lipids that are present in growth media are incorporated into membranes.Thus also yeast cells are affected by lipid peroxidation (Costa and Ferreina 2001).

1.5 Roles of cell components in resistance mechanism

Oxidative stress occurs as a result of excess levels of reactive oxygen species which may result from transition from aerobic to anerobic conditions; raise in mitochondrial respiratory chain activity; exposure to oxidants, xenobiotics or drugs; depletion in antioxidant defenses (Costa and Ferreina 2001). On the other hand, cells possess oxidant defense systems as well as stress response to cope with oxidative stress.

The cell has its own antioxidant defense mechanism that includes cell components, proteins and enzymes. All mechanism will be discussed in Section 1.5.1.

1.5.1 Cell membrane components

H_2O_2 is a membrane-permeable, diffusible molecule and is harmful to cells after being converted to more toxic compounds. (Schrader and Fahimi, 2006). Although it is generally accepted that H_2O_2 freely penetrates cell membranes, different experiments in both *S.cerevisiae* and *E.coli* have shown that plasma membrane permeability limits H_2O_2 diffusion and hence protects the cells against H_2O_2-induced oxidative stress (Bayliak, et al, 2007).

Membrane lipid composition of yeast is found to be important for oxidative stress resistance. Yeast cells that contain high level of saturated fatty acids are found to be more resistant than higher level of polyunsaturated fatty acids (Derek, 1998).

Carotenoids are membrane-bound protective antioxidant pigments that belong to isoprenoid family and are produced by plants, algae, bacteria and fungi. Carotenoids are effective antioxidants and scavenge singlet oxygen (Drabkova, 2005).

The *Saccharomyces cerevisiae* yap1 null strain showed high resistance levels against H_2O_2, paraquat, menadione, and UV light after transformation with a *Chlamydomonas reinhardtii* rpl10a gene due to increased carotenoid levels (Álvarez, et al, 2002).

1.5.2 Vacuole / Lysosome

In yeast, protein sorting, vacuole function and vacuolar acidification are core functions to obtain broad resistance of oxidative stress. After oxidative damage besides the increase of vacuolar proteolysis, also the genes encoding vacuolar proteases, protein sorting and vacuolar fusion are up regulated. (Costa, et al, 2007) Vacuolar proteolysis has major role in turnover of proteins, especially vacuolar Pep4 aspartyl protease increases to remove oxidized proteins. However, cells treated with H_2O_2 release Pep4 into cytoplasm and hence oxidized proteins may not be degraded by Pep4 in vacuole (Costa, et al, 2007).

1.5.3 Proteasome

Proteosomes are multicatalytic ATP-dependent protease complexes that break-down normal, damage, mutant or misfolded proteins into short peptides (Albert, et al, 2002; Costa, et al, 2007).

Each proteasome contains 20S core proteasome able to degrade proteins. They are ATP and ubiquitin independent and are not activated under oxidative stress conditions. On the other hand, ATP/ubiquitin-dependent proteasome complex with an apparent sedimentation coefficient of 26S are unable to degrade oxidized proteins, because ubiquitin-activating and conjugating enzymes are very sensitive direct oxidative inactivation. During oxidative stress applications and after treatment 20S proteoasome genes are upregulated (Costa, et al, 2007).

1.5.4 Peroxisome

Peroxisomes are organelles surrounded by single membrane and selectively import the substance from cytosol with the help of proteins located at its membrane. Peroxisomes belong to microbody family and contain different kind of enzymes that are responsible for fatty acid metabolism, flavin oxidation, disproportionation of superoxide radicals, alcohol detoxification and useful oxidative reactions (Alberts, et al, 2002; Keeton, et al, 2003).

As a result of nutritional and environmental conditions peroxisomes are able to proliferate and multiply themselves or are degraded by the cell (Schrader, et al, 2006).

Peroxisome and mitochondria are both responsible for respiration. Although peroxidase is unable to produce ATP like mitochondria; peroxisome joins the respiratory pathway to reduce O_2 to H_2O_2 which is further reduced to H_2O (Schrader and Fahimi, 2006).

1.6 Main antioxidant defense in yeast

Enzymatic and non-enzymatic defense systems cooperate with each other to reduce ROS. The function of proteins is catalytically reducing ROS through electronic transfer, and non-enzymatic defense systems are essential to keep an adequate redox balance in the different cellular sub-components.

1.6.1 Non-enzymatic defense systems

1.6.1.1 Glutathione

Glutathione is a tripeptide γ-L-glutamyl-L-cytinlglycine and acts as a radical scavenger and protects the cells from free radicals because of its free thiol group which is normally in reduced form (Mathews and Van Holde, 1990; Derek, 1998).

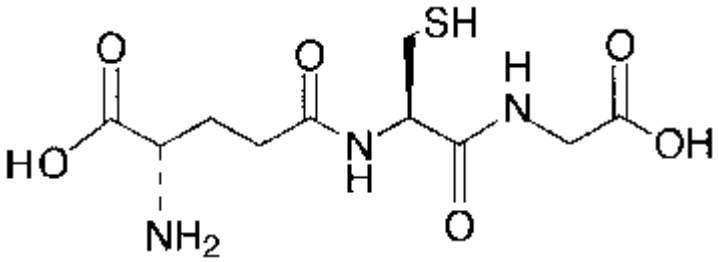


Figure 1.7 : Glutathione (Merck Index, 11th Edition**,** 4369).

Glutathione reduces disulfide bounds of cytoplasmic proteins non-enzymatically by acting as electron donor and glutathione is converted to oxidized glutathione (GSSG). Oxidized form of glutathione is reduced back to glutathione by glutathione reductase with NADPH-dependent manner.

$$GSSG + NAPH + H+ \longleftrightarrow 2GSH + NADP+$$

Reduction of peroxides is also carried out by glutathione (Mathews, et al, 1990).

In *S.cerevisae* glutathione biosynthesis is carried out by two different genes GSH1 and GSH2. GSH1 encodes γ-glutamylcysteine synthetase which catalyses the ligation of glutamate and cysteine aminoacids, whereas GSH2 encodes glutathione synthetase to add glycine to γ-glutamylcysteine (Sugiyama, et al, 2000).

Glutathione-deficent mutants were found to be hypersensitive to both H_2O_2, and superoxide anion generators such as menadione; but still able to induce adaptive response which would mean that GSH does not act as a sensor (Derek, 1998).

1.6.1.2 Polyamines

Polyamines (PAs) putrescine (Put), spermidine(Spd), spermine (Spm), and cadaverine (Cad) constitute a group of cell components that display high biological activity both in regulation of cell proliferation and cell differentiation. Polyamines are associated with anti stress protection of cells (Kuznetsov, et al, 2006).

Spermine and spermidine have a protective role in oxidative stress in *S.cerevisiae* and is also essential for *S.cerevisiae* aerobic growth. Additionally *S.cerevisiae* spe2 null mutants led to hypersensitivity to oxygen (Derek, 1998).

1.6.1.3 Trehalose

Dissaccharide trehalose is a soluble energy storage from in bacteria, yeast, fungi, higher and lower plants and invertebrate animals. Trehalose is composed of two glucose molecules bound by an alpha, alpha-1, 1 linkage (Mathews and Van Holde, 1990).

Different stress conditions cause trehalose accumulation depending on transcriptional activation of the trehalose-phosphate phosphatase gene, and stabilize proteins as a result of reducing aggregation of denatured proteins (Costa, et al, 2001).

Although, trehalose protects organisms against various stresses, such as dryness, freezing, and osmopressure, there are some doubts about its antioxidant properties. In several yeasts, there is no correlation found between levels of resistance to H_2O_2 and trehalose concentration of yeast. On the other hand to obtain resistance against oxidative stress, a threshold level of trehalose is found to be important for survival (Derek, 1998).

Yeast cells exposed to mild heat shock or proteasome inhibitor induce trehalose production and stress response, because these cells become resistant to hydrogen peroxide. Hydrogen peroxide-sensitive mutant cells show faster protein oxidation due to lack of trehalose content (Turanlı, 2003).

1.6.1.4 Metallothioneins:

Metallothionein is a family of low molecular weight, cysteine-rich proteins able to bind both essential and non-essential metals through the thiol group of their cysteine residues. This group of proteins is able to protect cells from metal stress and oxidative stress (Martinez, et al, 2006).

Metallothioneins are important to counter the toxicity of metals such as copper, cadmium, zinc, mercury, copper, arsenic and silver. Yeast metallothioneins are encoded by the CUP1 and CRS5 genes and that genes also protect the yeast cells against oxidants. Also oxidant-sensitive yeast strains that lack of Cu/Zn Sod are protected by the help of over-expression of yeast or human metallothionins (Derek, 1998).

1.6.1.5 Thioredoxin

Thioredoxins are sulphydryl-rich small proteins with an 12kDa that act as anti-oxidant with two thiol group in its active sites, Cys-Gly-Pro-Cys sequences. These two cysteines are the key to the ability of thioredoxin to reduce other proteins. (Mathews and Van Holde, 1990)

S.cerevisiae has two genes, TRX1 and TRX2 that encode thioredoxin proteins. *S.cerevisiae* that has deleted TRX2 genes is hypersensitive to H_2O_2 but not to diamide. Although deletion of both TRX1 and TRX2 genes is not lethal, they cause the extension of S-phase and increase the level of oxidised glutathione (Derek, 1998).

1.6.1.6 Glutaredoxin

Glutaredoxins like thioredoxins are small sulphydryl proteins that contain redox-active disulfide and act as source of electrons for ribonucleotide reductase (Mathews and Van Holde, 1990; Derek1998; Holmgren, 1989).

GRX1 and GRX2 are two different genes that encode highly similar proteins, but these proteins perform different roles to protect the yeast cells against hydrogen peroxide and superoxide anions (Derek, 1998).

Thioredoxin and glutaredoxins play a major role as cofactor for ribonucleotide reduction.Mutation studies in *E.coli* showed that mutants that lack both thioredoxin and glutaredoxin were unable to survive, but mutations in either thioredoxin or glutaredoxin did not inhibit cell survival (Mathews and Van Holde, 1990).

1.6.2 Enzymatic defense systems

In *S.cerevisiae*, different regulatory networks have several enzymes that remove the dangerous side-effects of oxygen radicals and their products or repair damages based on oxidative stress.

1.6.2.1 Catalase

Catalase protects the cell from the toxic effects of hydrogen peroxide by catalyzing decomposition into molecular oxygen and water without the production of free radicals. Catalase is a tetramer of four identical monomers that each of which contains heme as a prosthetic group at the catalytic centre (Mathews and Van Holde 1990).

The catalytic mechanism of catalase is thought to occur in two stages;

$$H_2O_2 + Fe(III)\text{-}E \rightarrow H_2O + O{=}Fe(IV)\text{-}E \quad (1)$$

$$H_2O_2 + O{=}Fe(IV)\text{-}E \rightarrow H_2O + Fe(III)\text{-}E \quad (2)$$

Figure 1.8 : The catalytic mechanism of catalase (Dounce, et al, 2004).

S.cerevisiae possesses two genes encoding catalase; CTA1 and CTT1. The CTA1 gene of *S. cerevisiae*, encodes catalase A, the peroxisomal catalase, and its main physiological role is to remove H_2O_2 by fatty acid β-oxidation.The other gene CTT1 encoding catalase T that knowns as the cytosolic catalase, is related to oxidative stress, osmotic stress and starvation (Derek, 1998).

S.cerevisae cytosolic or peroxisomal catalase deficient strains showed significant decrease in survival upon exposure to hydrogen peroxide (Baĭliak, et al, 2006).

Catalase genes are moderately inducible by hydrogen peroxide (Derek, 1998). The activity of catalase and superoxide dismutase increased with treatment of wild type cells with 0.5 mM H_2O_2 for 30 min. (Baĭliak, et al, 2006).

1.6.2.2 Superoxide dismutase

The enzyme superoxide dismutase belongs to metalloenzyme family and catalyzes dismutation of superoxide into oxygen and hydrogen peroxide (Mathews and Van Holde 1990).

S.cerevisiae expresses two forms of superoxide dismutase (SOD): MnSOD, encoded by SOD2, which is located within the mitochondrial matrix, and CuZnSOD, encoded by SOD1, which is cytoplasmically located. Cu/ZnSOD seems to be a major enzyme to remove superoxide anions from the cytoplasm and maybe peroxisomes. On the other hand, Mn/Sod only protects mitochondria from superoxide anions that result from respiration or exposure to ethanol (Derek, 1998).

1.6.2.3 Pentose Phosphate Pathway Enzymes:

Glucose-6-phosphate dehydrogenase(ZWF1), transketolase(TKL1) and ribulose-5-phosphate epimerase(RPE1) are involved in pentose phosphate metabolic pathway and mutation of these genes causes cells to become hypersensitive to oxidants such as H_2O_2. These genes are crucial for oxidative stress by cellular reducing power of NADPH (Derek, 1998).

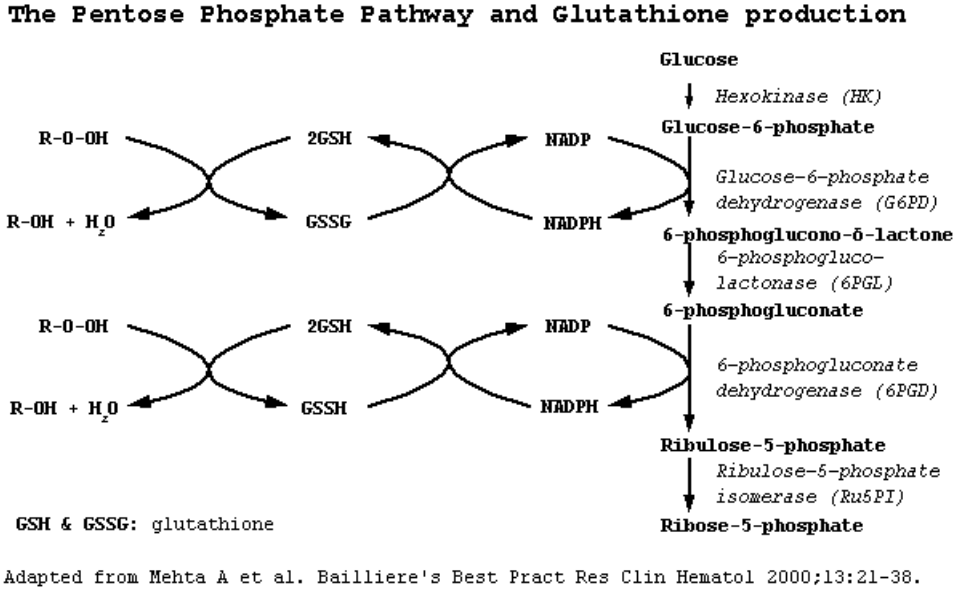


Figure 1.9 : The pentose phosphate pathway (Dounce, et al, 2004).

1.6.2.4. Glutathione reductase:

Glutathione(GSH) reduces non-enzymatically disulfide bonds of cytoplasmic proteins, and as a result it converts to its oxidised form(GSSH).Maintaining the intracellular GSH/GSSG ratio is obligatory for cell survival and the oxidized glutathione (GSSG) is reduced by glutathione reductase by the NADPHdependent manner to keep that ratio constant. An example reaction for glutathione reductase is shown in Figure 1.10.

$$GS–SG + \mathbf{NADPH} + H+ \rightarrow 2\ GSH + NADP+.$$

Figure 1.10 : Glutathione reaction mechanism. (Mathews, et al, 1990)

In *S.cerevisiae* GLR1 gene is responsible for coding glutathione reductase, and null GLR1 mutants were able to survive even though accumulation of oxidised glutathione caused hypersensitivity to oxidant (Derek, 1998).

1.6.2.5 Glutathione peroxidase:

Glutathione peroxidase is an selenium-containing enzyme that reduce H_2O_2 to water, along with oxidation of glutathione as well as lipid hydro peroxides to alcohols

$$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$$

Figure 1.11: Glutathione peroxidase reaction equation (Mathews, et al, 1990).

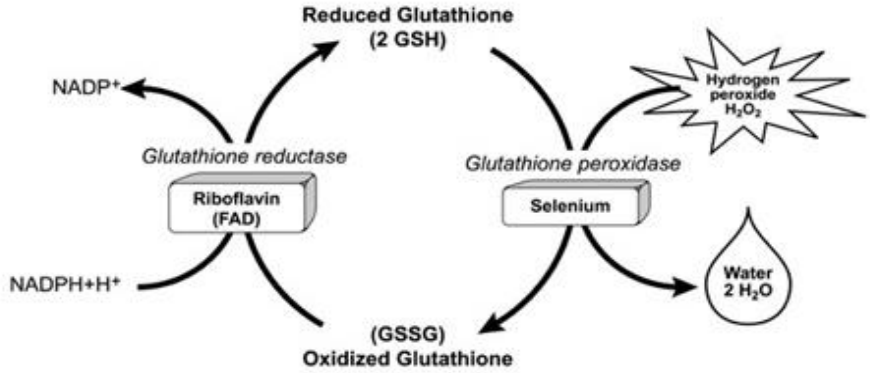


Figure 1.12 : Glutathione peroxidase reaction. (Mathews and Van Hold,, 1990)

Yeast glutathione peroxidase activity towards both hydrogen peroxide and organic hydroperoxides is induced by respiratory growth (Derek, 1998).

1.6.2.6 Thioredoxin peroxidase and thioredoxin reductase

The thioredoxin system is an important conserved system for yeast cells against oxidative stress and is composed of NADPH and three proteins, thioredoxin peroxidase (Tsa1p), thioredoxin (Trx2p), and thioredoxin reductase (Trr1p)(Holmgren, 1989).

Thioredoxin peroxidase uses a thioredoxin (Trx) as an immediate electron donor for the reduction of peroxide (Ross, et al, 2000). Thioredoxin is a small protein with two thiol groups and is able to reversibly oxidise and reduce to cope with oxidative stress (Mathews and Van Hold 1990). Thioredoxin reductase is a flavoprotein (FAD) enzyme that reduce thioredoxin (Trx) as well as other endogenous and exogenous compounds by NADPH- dependent manner (Mathews and Van Hold 1990; Mustacich, et al, 2000).

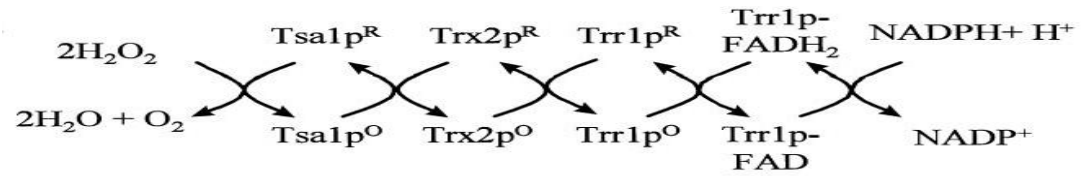


Figure 1.13 : Thioredoxin system. (Mathews and Van Hold 1990).

As a response to growth in 95 % O_2 or thiol containing agents thioredoxin peroxidase levels increased about two-fold in *S.cerevisiae* (Derek, 1998).

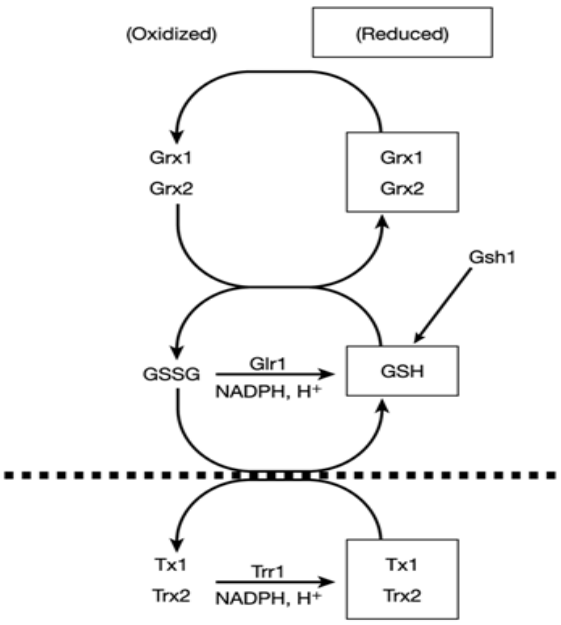


Figure 1.14 : Thioredoxin and glutaredoxin system relation (Mathews and Van Holde, 1990)

1.7 Hydrogen peroxide stress response

The budding yeast *S.cerevisiae*, is a single celled organism living freely in nature, hence it is faced with large variations in its natural environment. Survival of yeast depends on rapid response to sudden environmental changes and adaptation to these conditions.

Every cell has developed mechanisms to respond to changes in its environment and to adapt its growth and metabolism to unfavorable conditions. Two different responses are given by yeast cells. The first response is early response, also known as general response in which yeast cells adopt to stress conditions and include immediate protection of the cell against different sublethal stress conditions. In early response, yeast cells sense stress situation and then signal transduction pathways change gene expression activating both general and specific stress response, The second response is late response, also known as specific response, which involves synthesis de novo of stress proteins and antioxidants (Costa and Ferreina, 2001; Hohmann, et al, 2003).

Different transcription factors regulate yeast oxidative stress response. Yeast cells exposed to H_2O_2 give that general response via Msn2/Msn4 transcription factor, whereas specific response is mediated by Yap1p, Skn7p and Skn7p.Transcription factors are able to induce anti-oxidant and repair genes, or repress the others (Costa, and Ferreina, 2001).

Yeast cells generally respond with a common molecular defense mechanism to different stressors such as heat shock, oxidative stress, and osmolarity. The common response mechanisms to environmental changes can be identified as the general stress response mechanism (Costa and Ferreina, 2001).

The coordinate increases and decreases in the expression of the genes in general stress response were referred to as the environmental stress response (ESR). The ESR is initiated in response to a wide variety of environmental transitions, but precise levels and timing of the gene expression changes specific to the features of each new environment. Environmental stress response genes encoding both positive and negative regulators of Protein Kinase A (PKA) signaling are coinduced in the ESR (Hohmann; et al; 2003).

1.7.1 An H_2O_2-inducible Msn2/4 pathway

General stress response mechanism is achieved by a consensus five base pair element (CCCCT) called the "stress response element", or STRE. The Msn2p and Msn4p (Msn, multicopy suppressor of snf1) are two transcription factors that bind to STRE upon stress activation and induce gene transcription. Deletion of these transcription factors causes sensitivity to a variety of stress conditions that include oxidative stress (Demasi, et al, 2006; Hohmann; et al; 2003).

Although the target genes of Msn2/4 overlap different stress conditions to H_2O_2; they are not identical. The Δmsn2 Δmsn4 double deletion strain affects 180 genes that are induced by H_2O_2 and are hypersensitive to H_2O_2 (Hohmann; et al; 2003).

Msn2/4 is a negative control by the Ras-cAMP-Protein kinase A (PKA) pathway. In case of different stress conditions, cAMP levels and PKA activity decrease, and Msn2/4p mediated stress response is activated, Msn2/4 protein is then localized in the nucleus and that cause induction of their gene targets (Derek, 1998).

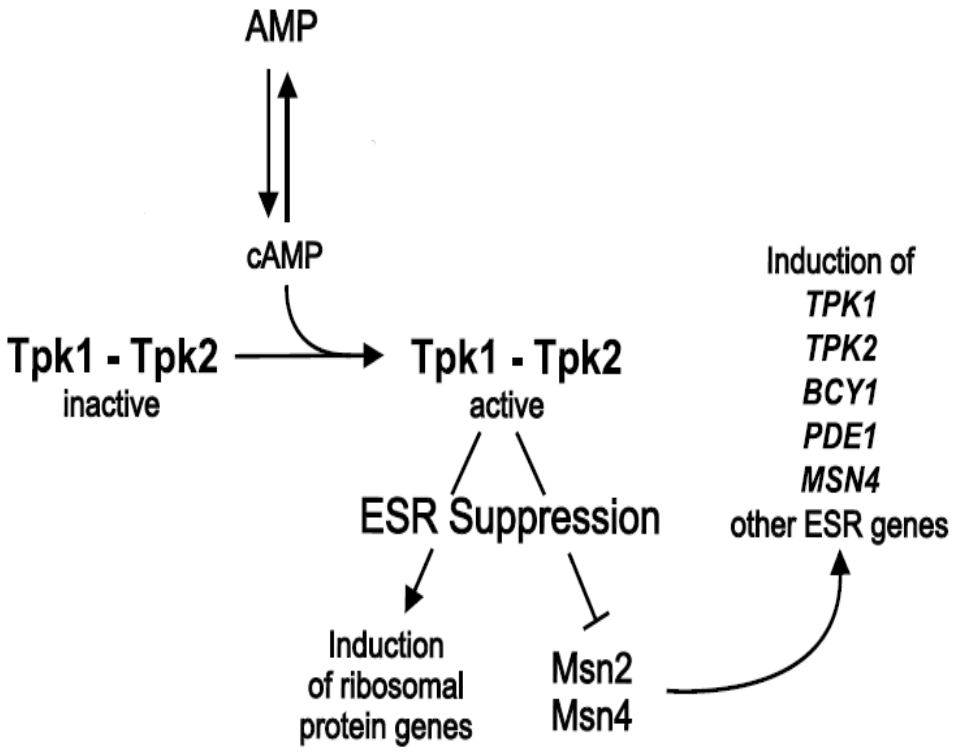


Figure 1.15 : Msn2/4 pathway. (Hohmann; et al; 2003)

1.7.2 An H_2O_2-inducible Skn7p Affects General Response

Skn7p is a transcription factor that is involved in trascriptional activation of heat shock genes in hydrogen peroxide response, through binding to heat shock elements. Thus, loss of Skn7p activity increases sensitivity to hydrogen peroxide. Skn7p co-operates with Yap1p in regulating expression of a set of genes related to oxidative defense (Demasi, et al, 2006; Hohmann; et al; 2003).

Yap1p/Skn7p mediates upregulation of thioredoxin system that contain thioredoxin peroxidase (Tsa1p), thioredoxin (Trx2p), and thioredoxin reductase (Trr1p). The reduction of Yap1p disulfide bonds by the thioredoxin system results in a nuclear export signal.Thus Yap1p is exported back to and accumulated in the cytoplasm (Lee, et al, 1999).

1.7.3 An H_2O_2-inducible Xbp1p Effects on General Response

Xbp1 is the only known stress-induced transcriptional repressor of the *Saccharomyces cerevisiae* in response to oxidative stress. XBP1 gene belongs to Swi4/Mbp1 family and is also responsible for various stress responses including heat shock, high osmolarity, and glucose starvation (Mai and Breeden, 1997).

Xbp1 is a 72 kDa protein and has a conserved helix-loop-helix domain which is responsible for DNA binding. Its promoter region contains five STRE, one HSE (heat shock element) and one ARE (Yap1p-binding site) (Derek, 1998).

1.7.4 An H_2O_2-inducible YAP1 Pathway

Yap1p is a bZip-type transcription factor and is the functional homologue of mammalian AP-1 in *S.cerevisiae.*(Rowley, et al, 1989) Yap1p activates expression of antioxidant genes in response to oxidative stress. Under non-stress conditions, Yap1p is localised in the cytoplasm, but when the cells are exposed to hydrogen peroxide, Yap1p rapidly accumulates in the nucleus (Azevedo, et al, 2003).

Yap1p contains two clusters of cysteine residues called cysteine-rich domains located at the N terminus (N-CRD) and the C terminus (C-CRD). In response to H_2O_2, Yap1 becomes oxidized to an intramolecular disulfide bond between N terminal Cys303 and C-terminal Cys598. Oxidant-specific folding of Yap1p regulates both transcriptional activation and nuclear localization of Yap1p (Gulshan, et al, 2005).

Nucleus is a membrane-enclosed organelle and its contents are separated from the cytoplasm. The nuclear membrane is impermeable to most molecules, thus the entry and exit of large molecules from the nucleus is tightly controlled by the nuclear pore. The movement of molecules is carefully controlled and maintained via active transport of molecules by cargo proteins. Cargo proteins can be divided into exportins and importins. Binding of a cargo to exportins facilitates its export to the cytoplasm and importins facilitate import into the nucleus (Moore , 1998).Yap1p transport from nuclear pore complex only requires Nuclear Export Signal(NES) (Kuge, et al, 1998).

Nuclear export of Yap1p requires nuclear export signal as well as nuclear export receptor (Crm1p).Under non-stress conditions, Crm1p binds to a nuclear export sequence (NES)-like sequence in Yap1p in the presence of Ran-GTP and due to active nuclear export of Yap1p, it is localized in the cytoplasm. On the other hand, stress conditions promote Yap1p nuclear accumulation by modification of its nuclear export sequence that causes inhibition of Yap1p/Crm1p interaction and Yap1p accumulates in the nucleus (Kuge, et al, 1998).

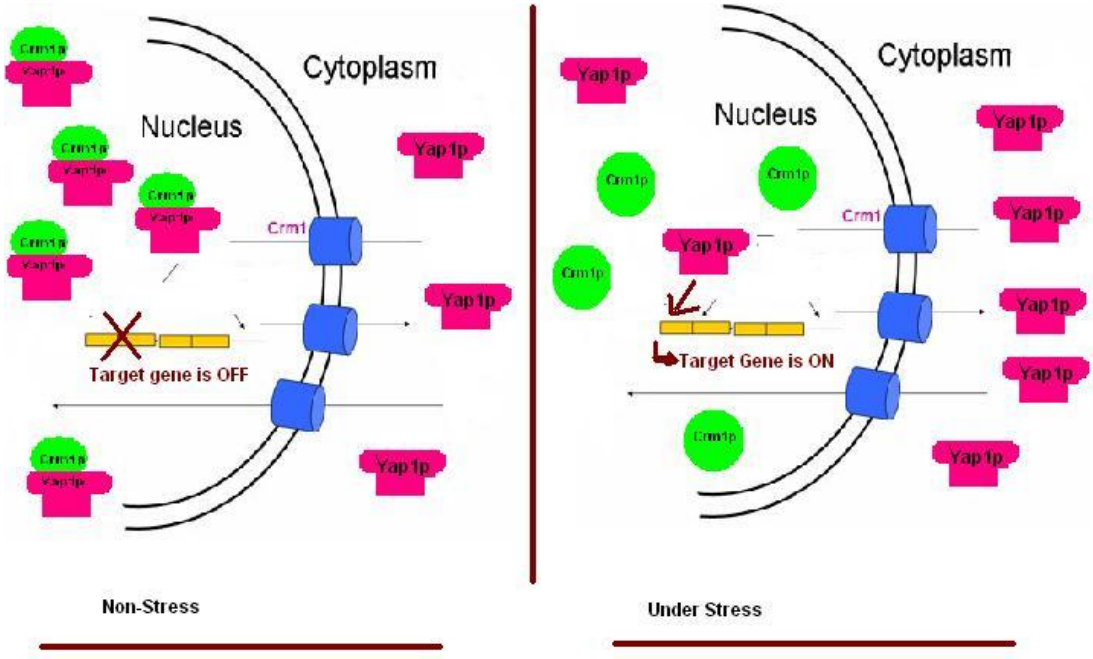


Figure 1.16 : Crm1p-Yap1p interaction under stress and non-stress conditions. (Kuge, et al,1998).

1.8 How to obtain hydrogen peroxide resistant *Saccharomyces cerevisiae*

Many strategies such as random mutagenesis, metabolic engineering, evolutionary engineering have been developed and implemented to engineer microorganisms and production processes to improve yield and productivity. Aim of all these strategies are overproduction of value added products with high yields. Metabolic and evolutionary engineering strategies are generally used for stress resistance studies in yeast (Çakar, et. al, 2005).

The metabolic engineering approach combines systematic analysis of metabolic and other pathways with molecular biological techniques to improve cellular properties by rational genetic modifications (Nguyen, 2005).

The metabolic engineering strategies are divided rational (forward) metabolic engineering and inverse metabolic engineering, and those strategies can complement each other in a strain development programme. Rational metabolic engineering is a conventional approach to cellular engineering that leans on direct perturbations of the metabolic network to understand the mechanism. Conversely, inverse metabolic engineering is a global approach to identify genetic loci that are important for a given phenotype (Nguyen, 2005).

In this thesis, evolutionary engineering, an inverse metabolic engineering strategy, was designed and employed to obtain oxidative stress resistant *S. cerevisiae* mutant yeasts. For that purpose EMS was used as a chemical mutagen which causes many mutations throughout the genome and increases the genetic diversity of the initial yeast population. After screening for oxidative stress resistance of the EMS mutant and the wild type yeast, the EMS mutagenized resistant yeast population was exposed to hydrogen peroxide pulses in liquid cultures in successive batch cultivations.

1.9 Aim of the Study

The first aim of the present study was to obtain hydrogen peroxide-resistant yeast strains. Evolutionary engineering strategy was used to obtain these yeast cells. Hydrogen peroxide-resistant generations were obtained by appling pulse stress to each generation. Randomly selected hydrogen peroxide resistant individuals were

screened for possible cross-resistance to other stress conditions. In this study, the second aim was to understand molecular mechanisms of response, repair and adaptation to hydrogen peroxide stres. Thus, the mutant individuals were also screened for catalase activity and expression levels of selected oxidative stress related genes “YAP1, CTT1, HSP104, AFT1, GLR1and YAP5” were determined in the absence and presence of H_2O_2. The hydrogen peroxide-resistant yeast cells would provide us useful information to understand oxidative stress mechanism that could further be used to prevent oxidative damage. On the other hand, further transcriptomic and proteomic analysis would provide us more detailed information about oxidative stres and related diseases such as cancer or ageing.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipments

2.1.1 Laboratory equipment

The equipments used in the study are given in Table 2.1:

Table 2.1 : Laboratory equipment used in the study

Equipment	**Supplier Company**
Centrifuge	Allegra 25R Centrifuge Beckman
Microfuge	Eppendorf Microcentrifuge (USA)
High pressure steam sterilizer	TOMY SX-700E
Magnetic stirrer	Labworld Standard Unit
pH Meter	Mettler Toledo MP220
Precision Balance	Precisa 620C SCS
Balance	Precisa BJ 610 C
UV Visible Spectrophotometer	Shimadzu UV - 1601
UV Transilluminator	Biorad UV Transilluminator 2000
Electrophoresis equipments	ThermoEC MiniCell® PrimoTM EC320 Electrophoretic Gel System
Power supply	EC250-90 Apparatus Corporation
Microwave	Arçelik MD582
Ice machine	Scotsman AF 10
Vortex	NuveNM 110 (Turkey)
Water Baths	Memmert, wb-22
Thermomixer	Eppendorf Thermomixer Comfort
Laminar air flow cabinets	FASTER BH-EN 2003
Thermomixer Compact	Eppendorf
Power supply	Thermo Electron Corporation EC250-90
Shaker	Certomat S-2
Freezers	-80 ^{o}C Heto Ultrafreeze 4410 -20^{o}C Arçelik, +4 ^{o}C Arçelik
Microplate Spectrophotometer	BIORAD Benchmark Plus
Pure water system	TKA Wasseraufbereitungssysteme
Pipettes	2.5µl, 10µl, 100µl, 200µl, 1000µl, Eppendorf
Multichannel (12 channel) pipette	50-300µl, Genex beta

2.1.2 Chemicals and enzymes

Chemicals and enzymes used in the study are shown in Table 2.2:

Table 2.2 : Chemicals and enzymes

Material	**Supplier Company**
MassRuler™ DNA Ladder (Mix, 80bp-10Kb) Mass Ruler Low Range DNA Ladder (80-1031 bp) 25 mM $MgCl_2$ 6X Loading dye	Fermentas
Low melting agarose TEMED	AppliChem
$NaH_2PO_4.2H_2O$ KH_2PO_4 K_2HPO_4 Absolute ethanol	J.T. Baker
Tryptone	Lab M ™
Albumine, bovine (BSA) Glycerol	Sigma
Tris Base Acrylamide	BDH Laboratory
Dimetinylsulfoxide(DMSO) $ZnCl_2$ Acetone	Riedel- de Haën
Glisin EDTA Glucose Ethidium bromide Yeast Extract Agar KCl HCl H_2O_2 $CoCI_2$	Merck
NaCl	CARLO ERBA

2.1.3 Commercial kits

Commercial kits used in this study are given in Table 2.3:

Table 2.3 : Commercial kits

Kit	**Supplier Company**
High Pure RNA Isolation Kit	Roche
SuperScript III One Step RT-PCR System	Invitrogen
Qubit	Invitrogen

2.1.4 Buffers and solutions

The compositions and preparation of buffers and solutions are given in Appendix A.

2.1.5 Software and Websites

Software and websites used in this study are given in Table 2.4:

Table 2.4 : Software and websites

Software / Website	**Supplier Company**
Bio – Capt	Vilber Loumat
UV - Spot	UVItec Ltd
Oligo Analyser http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/	Integrated DNA Technologies
Saccharomyces Genome Database http://www.yeastgenome.org	
UVIPhotoMW Version 99.05 for Windows 95 & 98,	UVItec Ltd

2.1.6 Yeast strains

The wild type strain used in this study *Saccharomyces cerevisiae* CEN.PK113.7D was kindly provided by Dr. Laurent Benbadis (INSA, Toulouse, France) and named as 905. *S.cerevisiae* CEN.PK 113-7D cells were mutagenized by ethyl methane sulphonate (EMS) (Lawrence et al., 1991), and mutagenized strains were named as 906. The, mutant yeast cell population was then exposed to increasing hydrogen peroxide (H_2O_2) pulse stress for selections.

2.1.7 Yeast Culture Media and Culture Conditions

2.1.7.1 Composition of yeast minimal medium (YMM)

Yeast Minimal Medium (YMM) was used in all experiments that contain the minimum amount of nutrients required for cell growth. It consisted of 2% (w/v) glucose and 0.67% (w/v) yeast nitrogen base without aminoacids. Cultures were grown at 30^{o}C and shaken at 150 rpm unless otherwise noted. The culture volume did not exceed 20% of the flask capacity.

2.1.7.2 Composition of xylose selective medium

During the experiments *S. cerevisiae* strains were growh in YMM; but to test any contamination, different generations were incubated in YMM with on initial OD_{600} of 0.03 which contained 2% (w/v) xylose instead of glucose, and 0.67% weight to volume yeast nitrogen base without aminoacids. OD_{600} value of the cultures incubated at 30^{o}C, 150 rpm were recorded for 72 h. The presence of growth in xylose-YMM indicated a possible contamination with other common yeasts such as *Pichia* and *Candida*.

2.2 Methods

2.2.1 Obtaining oxidative stress resistant yeast

2.2.1.1 Chemical mutagenesis

Ethylmethane sulfonate was used for random mutagenesis as described previously by Lawrence in 1991.

2.2.1.2 Screening of mutant populations under various oxidative stress conditions

To determine the lethal levels of hydrogen peroxide, for EMS mutagenized *S.cerevisiae,* the culture was inoculated into 10 ml YMM. Overnight incubation was performed at 30^oC and 150 rpm, the cultures were then exposed to 0 mM, 2 mM, 4 mM, 6 mM, 8 mM, 10 mM, 20 mM and 40 mM H_2O_2, when their A_{600} was 0.5-0.6 the pulse oxidative stress was applied for 90 min.

2.2.2 Pulse stress selection strategies for obtaining hydrogen peroxide resistant mutants

Pulse stress selection strategy was used to obtain hydrogen-peroxide resistant mutants. The stock culture was taken from the -80^0C freezer and inoculated into 10 ml YMM for overnight incubation. Thereafter, the cells were transformed to fresh YMM in 10 ml, the density of the culture was adjusted to OD_{600} 0.2 – 0.3 and inoculated at 150 rpm and 30^oC until the final optical density value (A_{600}) reached 0.5-0.6. At the end of the second incubation, 1 ml of culture was transferred to each of four microfuge tubes. All microfuge tubes were centrifuged at 10.000 rpm for five min. and the supernatants were discarded. Two of the tubes were chosen as control and filled with 1 ml of YMM.

The other two microfuge tubes were filled with YMM containing 5 mM H_2O_2. All tubes were inoculated during 90 minute at 150 rpm and 30^oC. At the end of 90 min, all microfuge tubes were centrifuged at 14,000 rpm for five min and washed twice with 1 ml YMM. Both control and H_2O_2 exposed yeast cells were inoculated at 30^oC in 10 ml YMM for 16 h. At the end of 16 h of incubation, OD_{600} value was used for survival assay. The survival assay measures the ability of cells to survive upon exposure to H_2O_2. The survival assay data results were used for each generation to

determine selection conditions.The H_2O_2 stress levels were increased until the survival value of the tested generaton fel to 0.15.

2.2.3 Stock culture preparation

Frozen stock cultures of *S. cerevisiae* were prepared by mixing equal volumes of culture and 60% (v/v) glycerol in sterile 1.5 ml-tubes. Stock cultures were prepared aseptically and kept at -80^{o}C.

2.2.4 Selection of individual mutants from the final populations

The final generation exposed to 50 mM H_2O_2 (46th) were inoculated overnight in 10 ml YMM at 30^{o}C and 150 rpm without any stress condition. The sample was appropriately diluted upto 10^{-6} and a small aliquot was transferred to a YMM agar plate. The yeast cells were distributed over the surface. At the end of 48 hour of incubation, fifteen colonies were randomly choosen from the plate with toothpicks and transferred into 10 ml YMM for 16 h. incubation at 30^{o}C. Stock cultures were prepared for each selected individual at the end of that period.

2.2.5 Determination of resistant differences of individual mutants

Mid-log individuals (A_{600}, 0.3) and 905were grown approximately for 90 min until A_{600} of individuals reached to 0.5-0.6.Thereafter, both individuals and 905 were exposed to 50 mM H_2O_2 for 90 min. At the end of 90 min, individuals and 905 were washed with fresh YMM without dextrose to remove H_2O_2 effects and were resuspended in 1 ml fresh YMM. Both individuals and 905 were diluted to 10^{-5} and 5 µl of each dilution were inoculated to YMM agar plate for 48 h incubation at 30^{o}C.

2.2.6 Quantitative resistance determination of mutant individuals with the highest H_2O_2 resistance using high throughput screening method (Most probable number 'MPN')

Most probable number (MPN) method was used to estimate resistance of individuals and compare resistance in pulse or continuous stress conditions. At the first step, 180µl YMM were added into the first five wells of the 96-well plate, 20 µl sample were then placed into the first row of these five columns and mixed. In that way 1:10 dilution was obtained.That step was repeated by transferring 20 µl of preculture of sample to next row and mixed until the last row (8th row). Repeated dilution and

replication of cultures across several serial dilution steps resulted to up to 10^{-8} dilution. That step was repeated for each individual. The microbial growth indicated presence of microorganisms.

Two different stress conditions were applied during the 5-tube MPN assay to determine H_2O_2 resistance of mutant individuals. In the first condition; 905, final population and all individuals were exposed 50 mM H_2O_2 pulse stress. In the second condition 1 M and 2 M H_2O_2 were applied continuously to all individuals, 905 and final population as well.

2.2.6.1 Determination of resistance to pulse oxidative stress

Overnight liquid cultures of selected resistant individuals were diluted between A_{600} 0.2–0.3 and inoculated at 150 rpm and 30^{o}C until OD_{600} of 0.5-0.6. Hydrogen peroxide was then applied during 90 min. at 150 rpm and 30^{o}C. At the end of 90 min, individuals were washed twice with YMM, 20 µl samples from each individual was replaced into the wells that contain 180 µl YMM. In each step, 20 µl samples was mixed with YMM and transferred to the next row repeatedly until the final (8th) row. Cultures were incubated at 30^{o}C for 72 h. and visible growth of each well was recorded at 24, 48 and 72h.

2.2.6.2 Determination of resistance to continious oxidative stress

Overnight liquid cultures of selected resistant individuals were diluted between A_{600} 0.2–0.3 and inoculated at 150 rpm and 30^{o}C until OD_{600} reached to 1.2-1.4. All individuals, 905 and the final population were exposed to two different hydrogen peroxide levels. (1M and 2 M H_2O_2).

2.2.7 Determination of other stress resistances

The selected individuals shown in Table 3.3 were used for all cross-resistance experiments. The selected individuals; final population and the wild type strain "905" were exposed to pulse or continuous stresses depending on the stress type and level.

905 and all selected individuals were screened on YMM agar plates that contained different kinds of stress agents to determine their potential cross-resistances to corresponding stresses. That was the initial step to determine cross-resistance and

quantitative analysis of the stress resistance was performed later by pulse or continuous 5-tube MPN methodology.

In pulse test strategy, overnight grown cultures were diluted appropriately as described previously and incubated at 30°C, 150 rpm until they reached early or mid-exponential growth phase. At this step, one ml of each sample was taken and washed with dextrose-free YMM and exposed to the stress agent for the time specified (60 or 90 min.). All samples were then centrifuged for 5 min at 14,000 rpm the supernatant was discarded. All samples were washed twice with YMM without dextrose and centrifuged again for 5 min. at 14,000 rpm. Their supernatants were discarded and the pellets were resuspended in 1 ml YMM. Twenty µl of the sample were delivered into the first row of the five columns of 96-well plate, filled with 180 µl YMM. Several dilutions were applied until the last row. For each sample, control groups were not exposed to any stress agent, but all other steps were also applied to them.

In continuous test strategy; the selected individuals, final population and wild type strain "905" were incubated at 30°C, 150 rpm untill they reached early or mid exponential growth phase. Thereafter, 1 ml of each sample was taken into the microfuge tube and washed with dextrose-free YMM and centrifuged for 5 min. at 14,000 rpm. The supernatants of all samples were discarded and resuspended in 1 ml fresh YMM. 20 µl of the sample were delivered into the first row of the five columns of the 96-well plates that were filled with 180 µl YMM with stress agent. As a control group 20 µl of the sample were delivered into and another first row of the five columns that contained YMM without stress agent.

2.2.7.1 Application of heat stress

The heat stress response of wild type 905, final populations and all individuals was studied by applying pulse heat stress. All samples were exposed to 60^0C stress for 10 min. The cultures with 30 ^{0}C exposure were used as the control group. Control and experiment groups' viable cell numbers per ml and their survival ratio were determinated by using 5-tube MPN methodology.

2.2.7.2 Application of freezing thaw

The freeze-thaw stress response of wild type 905, final populations and all individuals was studied by pulse stress tests. One ml of overnight cultures of all samples were taken and washed with dextrose-free YMM and exposed to -20^0 C temperature stress for 90 min and -196^0C temperature stress for 10 min. The cultures with 30 ^{0}C exposure were used as the control group. Control and experiment groups' viable cell numbers per ml and their survival ratio were determinated by using 5-tube MPN methodology.

2.2.7.3 Application of ethanol stress

The ethanol stress response of wild type 905, final populations and all individuals was studied by continuous stress strategy. All samples were exposed to 5 and 7% (v/v) ethanol stress. Control and experiment groups' viable cell numbers per ml and their survival ratio were determinated by using 5-tube MPN methodology.

2.2.7.4 Application of iron stress

The ferro stress response of wild type 905, final populations and all individuals was studied by continuous stress test. All samples were exposed to 2 mM $FeCI_2$ and 5mM $FeCl_2$. Control and experiment groups' viable cell numbers per ml and their survival ratio were determinated by using 5-tube MPN methodology.

2.2.7.5 Application of copper stress

The copper stress response of wild type 905, final populations and all individuals was studied by continuous stress test. All samples were exposed to 0.25 mM $CuCl_2$. Control and experiment groups' viable cell numbers per ml and their survival ratio were determinated by using 5-tube MPN methodology.

2.2.8 Growth curve analyses of best individuals obtained from different pulse oxidative stress conditions

The growth physiology of the wild type and the mutant individuals with highest H_2O_2 resistance, "B1, B11 and B14", was determined in the presence and absence of 1 mM H_2O_2 in the culture medium containing YMM. During the experiment all samples were inoculated to 200 ml YMM in 2 L flasks. At 0^{th}, 3^{nd}, 6^{th}, 9^{th}, 12^{th}, 18^{th},

21^{th}, 24^{th}, 27^{nd}, 30^{th}, 36^{th}, and 45^{th} h. of incubation, samples were taken for spectrophotometric measurements.

2.2.9 Determination of enzyme activity

2.2.9.1 Preparation of cell extracts

After overnight incubation of the wild type and the mutant individual with highest resistance, "B1,B11 and B14", at 30^0C and 150 rpm in YMM with and without 1 mM H_2O_2. One ml of each sample was transferred into a microfuge tube when their OD_{600} value reached the value at which the highest catalase activity was obtained. All samples were harvested by centrifugation at 14,000 rpm for 5 min. at room tempature, and the supernatant was discarded. The pellets were washed twice with the same amount of distilled water and resuspended in 200-250 µl phosphate buffers. (50 mM, pH 7.2). Yeast cell disruption was carried out by agitation with sterile glass beads. Thus the samples were enclosed in microfuge tubes as a final volume of 1 ml and wrapped by parafilm. The tubes were vortexed and placed to -80˚C to freeze and were thawn for 1 min. These steps were run repeated for over ten times. The cell extracts were replaced into a new microfuge tube, and centrifuged for 10 min. at 12,000 rpm (4˚C). The supenatant was used in catalase activity and total soluble protein determination assays.

2.2.9.2 Catalase enzyme activity assay

The catalase enzyme activity assay was quantified based on either the decrease in absorbance of hydrogen peroxide at λ = 240 nm. The prepared cell extract was used to perform catalase enzyme activity assay. 40 µl cell extract was added directly into the reaction mixture that contained 680 µl phosphate buffer and 480 µl 40 mM H_2O_2. The reaction mixture was incubated at 30˚C for 2.5 minute before yeast extract was added. The quartz cuvettes were then immediately scanned in a spectrophotometer at λ = 240 nm every 10 sec for 2 min. The catalase activities of yeast extracts were normalized to total cellular protein concentration in the lysate. One unit of enzyme activity was expressed as Δ E/min/mg protein.

2.2.9.3 Determination of protein contents

The protein concentration in the cell lysates was determined using the standard Bradford colorimetric assay, with bovine serum albumen as the standard according to Bradford, 1976. The measurement was performed at 595 nm using a Microplate Reader 3550-UV (Biorad).

2.2.10 Expression analysis

2.2.10.1 RNA isolation from yeast cells

Total RNA of the wild type and the resistant individuals were required for gene expression analysis with reverse transcriptase PCR. For this purpose the total RNA was isolated from the yeast cells via commercial kits (High Pure RNA Isolations Kit from Roche). The protocol used for High Pure RNA Isolation is described beow:

- The wild type and theresistant individual cultures in YMM were harvested during the early-stationary phase of growth, (OD_{600} 3.5-4.5). A dilution which gives a A_{600} of 1/ml was used.
- The samples were collected by centrifugation at 2,000 g for 5 min. in a standard table-top centrifuge.
- The supernatant was removed and the pellet was resuspended in 200 µL sterile PBS and transferred to a 2 mL microfuge tube.
- 10 µL of Lyticase (0.5 mg/ml) was added onto each tube, and the tubes were incubated at 30 °C for 15 min.
- 400 µL Lysis/Binding buffer was added to the microfuge tube and vortexed for 15 sec.
- A High Pure filter tube was inserted into a collection tube and the mixture was added on the upper reservoir of the column.
- The tubes were centrifuged at 8,000 g for 15 sec.
- The liquid collected in the collection tube was discarded.
- 150 µL DNase solutions per sample were mixed in a separate tube (135 µL DNase incubation buffer and 15 µL DnaseI). That mixture was incubated at 15 − 25 °C for 15 min.
- 500 µL Wash Buffer I was added on the filter tubes and centrifuged at 8,000 g for 15 sec., and the fluid was discarded.

- 500 µL Wash Buffer II was added on the filter tubes and centrifuged at 8,000 g for 15 sec., and the fluid was discarded.
- 200 µL Wash Buffer II was added on the filter tubes and centrifuged at maximum speed for 2 min., and the fluid was discarded with the collection tube.
- The filter tube was inserted into a 1.5 mL microfuge tube.
- Approximately 80 µL of elution buffer was added to the filter tube and centrifuged at 8,000 g for 1 min.
- The isolated RNAs were used as templates for cDNA synthesis reaction; used RNAs were stored in −80 °C for later usage.

2.2.10.2 Determination of RNA Concentration

Total RNA concentration of samples was measured by Qubit fluorometer from (Invitrogen). The kit contained buffer, an assay-specific dye, and high and low standards that were prepared with a fixed amount of RNA and combined with buffer and dye. The samples desired to be quantified were prepared similarly using 1-20 µl of each sample combined with buffer and dye. All samples were then incubated at least 2 min. before mesurement was performed. To fit a standard curve high concentration standard and low concentration standard were first measured.Thereafter, with the help of the parameters from that measurement; the quantities of all samples were astimated using the fluorescence of the samples and linear regression.

2.2.10.3 cDNA Synthesis and RT-PCR

To determine the expression levels of different genes related to oxidative stress, RNA of the wild type and highest resistant individuals were isolated from cultures in the absence and presence of 1 mM H_2O_2 in YMM. The cDNA was then synthesized from isolated total RNA via first two steps of SuperScript III One Step RT-PCR System (Invitrogen). The optimal temperature for reverse transcription was determined as 55^oC depending on primer and target sequences. The latter steps were performed for PCR amplification of isolated total RNA from yeast cells. The PCR cycle conditions were modified according to the desired amplicon (Table 2.5). The differences in PCR cycle conditions were in annealing temperatures, caused by the different GC content of primer sets.

Table 2.5 : RT-PCR cycles

	Temperature (ºC)	**Time**	**Cycle number**
cDNA synthesis	55	30 min	1
Denaturation	94	2 min	1
PCR			
Denaturation	94	15 sec	**36**
Annealing	Depend on Primer	90 sec	
Extension	68	50 sec	
Final extension	68	5 min	1

2.2.10.4 Oligonucleotide Primers

The oligonucleotide primers used in this study were selected from previous studies and are given in Table 2.6.They were below and confirmed on Amplify 3X software to check that primers actually bind their target sequence on desired gene. The efficiency of binding and the amplicon sizes are also determined with this software.

It is also important to analyze the primer sets for dimer formation. The hairpin, heterodimer and self dimer analysis of the primer sets are done and confirmed with the SciTools on the IDT DNA website.

Table 2 6 : Oligonucleotide primers

Gene	**Primer Sequence**	**Amplicon Size**
CTT1	F-5'-TGCAAGACTTCCATCTGCTG -3' R-5'-ACGGTGGAAAAACGAACAAG -3'	193 bp
YAP1	F-5'-TACACGTGATGGCGAGGATA -3' R-5'-TCCACTTCATTTTGCTGCTG -3'	210 bp
HSP104	F-5'-AGCAGGCTCGTCAAGGTAAA -3' R-5'-TAGTGGGAACGTCATCGTCA -3'	183 bp
AFT1	F-5'-ATGCATCTAAAAGGCCATGC -3' R-5'-GGCAGTGGCAAGATTTCATT -3'	195 bp
GLR1	F-5'- CCCCAGCGTAATTTCTCAC-3' R-5'-GTGCAGACCGACAACCTTTT -3'	360 bp
YAP5	F-5'- CGGAGGAAAAGAGTTCATCG -3' R-5'- TCATTTTGGAGGCGGTTTAG -3'	209 bp

2.2.10.5 Agarose gel electrophoresis

Low melting agarose gels (1.5%) were used to estimate the size of DNA molecules following PCR reaction. The DNA fragments obtained from PCR reaction were mixed with 6X loading dye in the proportion of 6:1 and placed into a well on the agarose gel. For the fragment size control, DNA markers were used. Electrophoretic separation was achieved by constant current at 120 mV for 30 min.

DNA within agarose gels were visualized under UV light by staining with ethidium bromide. The gel was placed into an UV illuminator that emits UV light at 302 nm and was photographed with a camera connected to a computer. Image files were saved with UVIPhotoMW Version 99.05 for Windows 95 & 98, UVItec Ltd. and subsequently analyzed. The size of the DNA was determined by comparing their mobility with the fragments of the DNA ladder.

3. RESULTS

3.1 Evolutionary Engineering of Oxidative Stress Resistant Mutant Yeast Cells

3.1.1 Screening of the wild type and the initial culture for determination of initial hydrogen peroxide stress levels

Initial stress conditions were determined by screening different H_2O_2 molarities. Depending on the previous information that *S. cerevisiae* 905 strain able to live in 2mM H_2O_2, the stress range screening procedure was started at 2 mM H_2O_2 and the strain exposed to 0 mM, 2 mM, 4 mM, 6 mM, 8 mM, 10 mM, 20 mM and 40 mM H_2O_2, respectively. During the screening; the optical density values of both the stress and the control groups were determined at 600 nm for 16^{th}, 18^{th}, 24^{th}, 41^{th} and 48^{th} hour of incubation. The quantitative data showing the comparison of survival ratio for each individual exposed to different H_2O_2 stress levels depending on incubation time are given in Table 3.1 and Figure 3.1.

Table 3. 1 : Comparison of survival ratio for each individual that exposed to different H_2O_2 stress levels of different durations.

SAMPLES	At 16. HOUR	At 18.HOUR	At 24.HOUR	At 41.HOUR	At 48.HOUR
2 mM H_2O_2	0.68	0.88	1.03	0.91	0.89
4 mM H_2O_2	0.07	0.04	0.62	0.97	0.96
6 mM H_2O_2	0.32	0.52	0.86	0.90	0.895
8 mM H_2O_2	0.13	0.12	0.81	0.88	0.90
10 mM H_2O_2	0.22	0.33	0.91	0.83	0.87
20 mM H_2O_2	0.03	0.17	0.36	0.91	0.90
40 mM H_2O_2	0.06	0.02	0.58	0.90	0.85

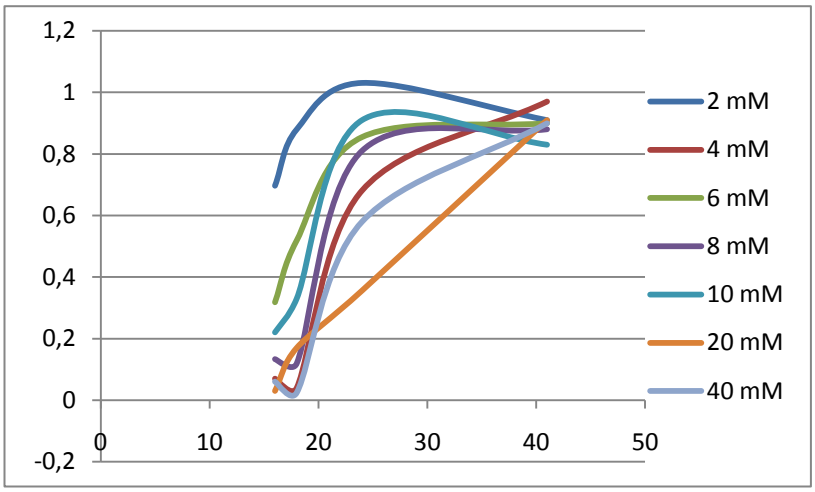


Figure 3. 1 : Comparision of survival ratio for each individual which exposed to different stress conditions depending on time.

According the data shown on Table 3.1; 5 mM H_2O_2 was chosen as an initial stress level and will be increased by 1 mM H_2O_2 for each generation.

3.1.2 Stress Application and Obtaining the Generations

The yeast cells exposed to stress regulate themselves for genetic make-up and growth. If the cells survive after first shock its physiology changes and they adapt to new conditions. Cells both repair damage and at the same time adapt to the environment to decrease the effects of stress. Depending on the success, cells either survive or die. Therefore, according to genetic possibilities of adaptation and energy supply, cells have a chance of adaptation. The last stage is the adaptation to growth. If it does not occur, the adapted cells eventually die (Smits and Brul, 2005).

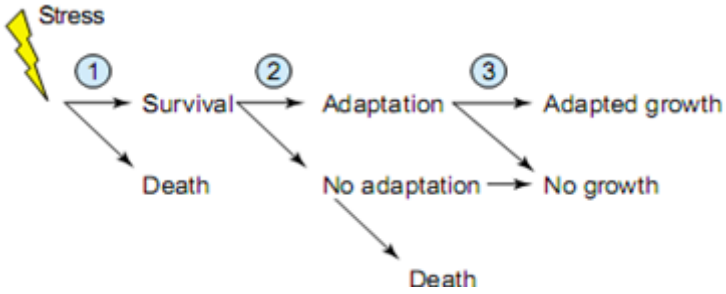


Figure 3.2 : Representation of the survival after stress conditions (Smits and Brul 2005).

During the experiments OD_{600} measurements' were used to understand whether or not cells could survive the stress. The first generation was exposed to 5 mM H_2O_2; which was increased by 1 mM H_2O_2 at each step. Survivors of each step were transferred to the next stress level. At the end of the 46^{th} increasing pulse stress

generation resistant to 50 mM H_2O_2, H_2O_2 levels higher than 50 mM H_2O_2 were too strong for the cells to survive. Thus, generation 46 was accepted on the final generation. The data of H_2O_2 response of yeast cells after exposure to different stress was collected and shown on Table 3.2 and Figure 3.3. Survival ratio of each generation is used to determine stress response of each generation.

Table 3.2 : Nomenclature for increasing-pulse stress populations and comparison of survival ratio for each generation at the selection stress level.

		OD_{600}		
Generations	**Concentration**	**Control**	**Stress**	**Survival Ratio**
G1	5 mM H_2O_2	5.66	4.40	0.78
G2	6 mM H_2O_2	4.85	3.68	0.76
G3	7 mM H_2O_2	5.13	2.60	0.51
G4	8 mM H_2O_2	3.09	2.05	0.66
G5	9 mM H_2O_2	5.93	3.78	0.64
G6	10 mM H_2O_2	4.52	4.13	0.91
G7	11 mM H_2O_2	4.78	1.61	0.34
G8	12 mM H_2O_2	5.48	1.97	0.36
G9	13 mM H_2O_2	7.73	5.46	0.71
G10	14 mM H_2O_2	5.49	4.18	0.76
G11	15 mM H_2O_2	3.97	3.58	0.90
G12	16 mM H_2O_2	4.80	2.72	0.57
G13	17 mM H_2O_2	4.65	2.40	0.52
G14	18 mM H_2O_2	7.43	5.21	0.70
G15	19 mM H_2O_2	6.92	4.62	0.67
G16	20 mM H_2O_2	5.47	4.14	0.76
G17	21 mM H_2O_2	7.02	2.25	0.32
G18	22 mM H_2O_2	6.99	3.53	0.50
G19	23 mM H_2O_2	4.545	2.27	0.50
G20	24 mM H_2O_2	5.05	1.99	0.40
G21	25 mM H_2O_2	8.94	3.37	0.38
G22	26 mM H_2O_2	3.57	1.23	0.34
G23	27 mM H_2O_2	3.72	1.73	0.46

Table 3.2 (contd.) : Nomenclature for increasing-pulse stress populations and comparison of survival ratio for each generation at the selection stress level.

G24	28 mM H_2O_2	4.26	1.23	0.29
G25	29 mM H_2O_2	2.25	0.84	0.37
G26	30 mM H_2O_2	3.49	1.15	0.33
G27	31 mM H_2O_2	2.67	1.05	0.39
G28	32 mM H_2O_2	3.82	1.21	0.32
G29	33 mM H_2O_2	2.90	1.05	0.36
G30	34 mM H_2O_2	3.45	1.15	0.33
G31	35 mM H_2O_2	5.83	1.79	0.31
G32	36 mM H_2O_2	2.86	0.72	0.25
G33	37 mM H_2O_2	4.97	1.10	0.22
G34	38 mM H_2O_2	2.83	0.72	0.25
G35	39 mM H_2O_2	3.30	0.76	0.23
G36	40 mM H_2O_2	3.60	0.63	0.18
G 37	41 mM H_2O_2	4.18	0.71	0.17
G38	42 mM H_2O_2	3.52	0.70	0.20
G39	43 mM H_2O_2	7.94	1.63	0.21
G40	44 mM H_2O_2	5.30	1.04	0.20
G41	45 mM H_2O_2	5.44	0.97	0.18
G42	46 mM H_2O_2	4.27	0.93	0.22
G43	47 mM H_2O_2	4.82	0.87	0.18
G44	48 mM H_2O_2	3.76	0.57	0.15
G45	49 mM H_2O_2	3.37	0.58	0.17
G46	50 mM H_2O_2	5.21	0.72	0.15
G46	50 mM H_2O_2	6.39	0.90	0.14

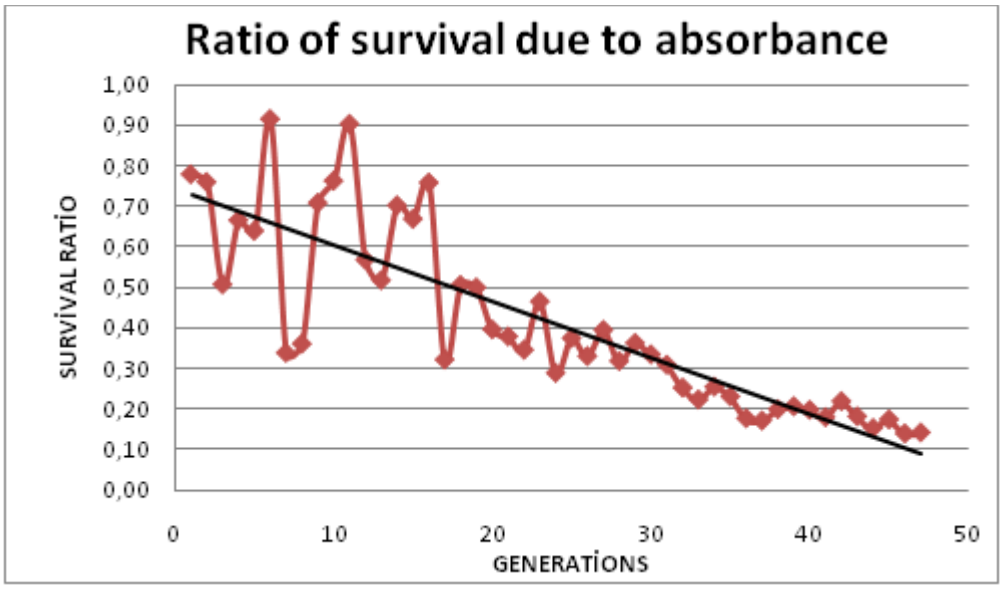


Figure 3.3 : Survival analyses of increasing stress generations.

3.1.3 Selection of individual mutants from final mutant populations

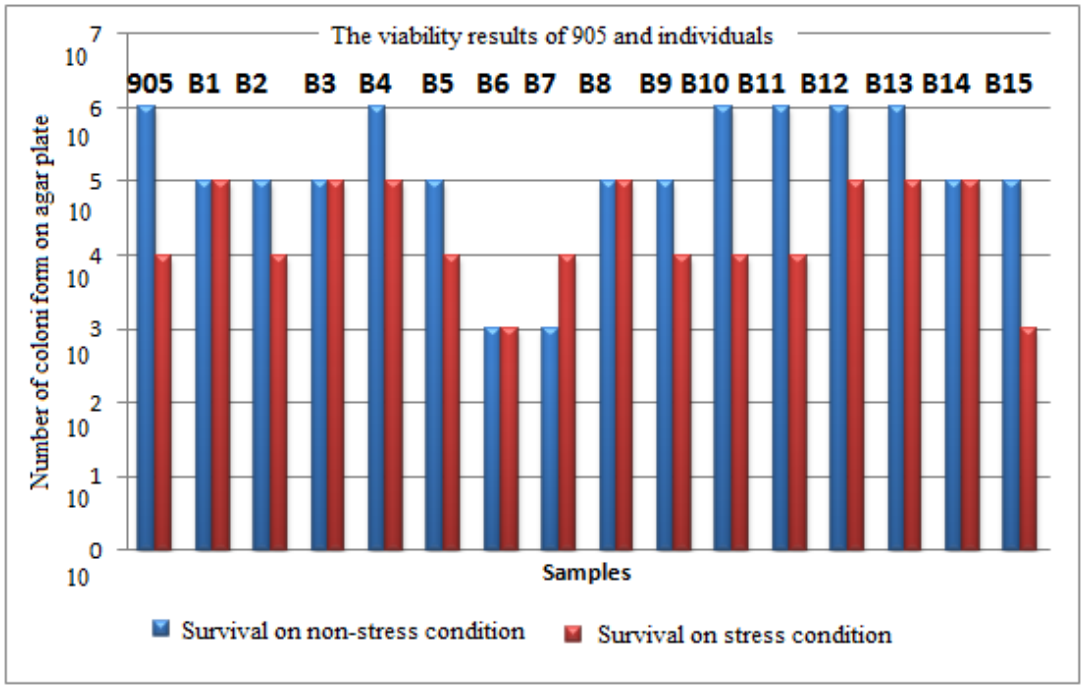
After an H_2O_2 resistant population was obtained; the individuals was selected from final population. For that purpose the final generation was inoculated onto YMM-agar plate in different dilutions. After incubation of YMM-agar plates fifteen colonies from 10^{-6} diluted plate was chosen randomly and inoculated into liquid YMM medium.

Table 3.3 : Nomenclature for mutant individuals from selected increasing stress final populations.

Individual mutant	Code
1st increasing stress individual	**B1**
2nd increasing stress individual	**B2**
3rd increasing stress individual	**B3**
4th increasing stress individual	**B4**
5th increasing stress individual	**B5**
6th increasing stress individual	**B6**
7th increasing stress individual	**B7**
8th increasing stress individual	**B8**
9th increasing stress individual	**B9**
10th increasing stress individual	**B10**
11th increasing stress individual	**B11**
12th increasing stress individual	**B12**
13th increasing stress individual	**B13**
14th increasing stress individual	**B14**
15th increasing stress individual	**B15**

3.1.4 Determination of resistance differences of individual mutants

The viability results of 905 and all individuals after 72 hours of incubation are shown above. All of the mutants were shown to be viable at less than 50 mM H_2O_2. However, wild type cells were also able to tolerate this hydrogen peroxide stress. The results of wild type and selected individuals after 50 mM H_2O_2 pulse stress application on the agar plate are shown on Figure 3.4.



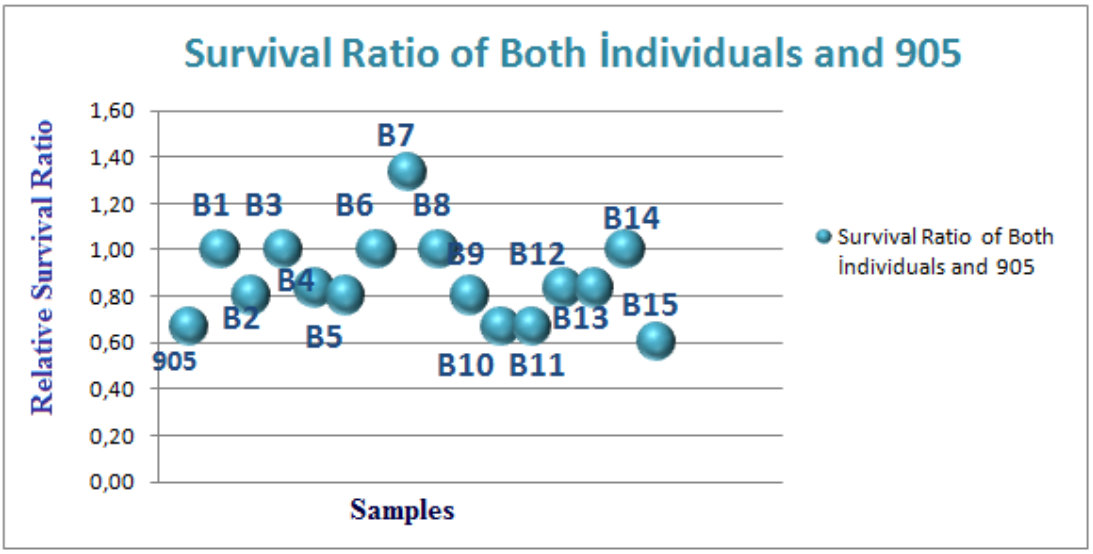


Figure 3.4 : Comparative control and stress values of both individuals and wild type.

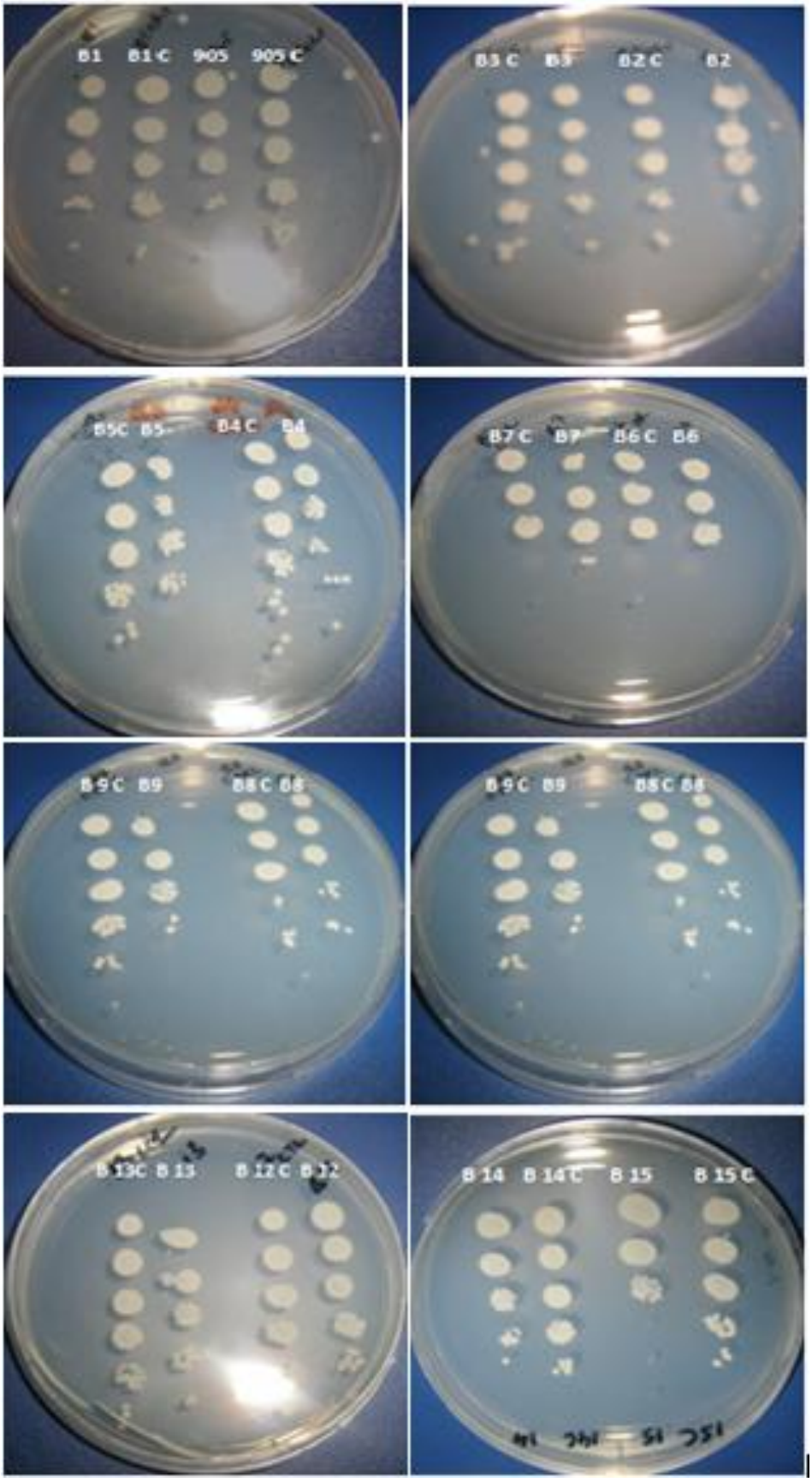


Figure 3.5 : The wild type and selected individuals after 50 mM H_2O_2 pulse stress application on the agar plate.

3.2 Determination pulse and continuous oxidative stress resistance of individuals

3.2.1 Continuous stress resistance of individuals

Overnight liquid cultures were diluted between OD_{600} 0.4-0.5 and inoculated at 150 rpm at 30^0C then all individuals, 905 and final population were exposed 0.1 M H_2O_2 and 0.2 M H_2O_2. The results of 72^{th} hours of incubation belong to 0.1 M H_2O_2 and 0.2 M H2O2 are shown on the Table 3.4.

Table 3.4 : Survival ratios of mutant individuals and wild type upon 0.1M H_2O_2 and 0.2 M H_2O_2 continuous stress application (After 72 h)

Individuals	Number of cells per ml (Control)	Number of cells per ml 0.1M H_2O_2	Number of cells per ml 0.2M H_2O_2	Survival Ratio as a Fold of Wild Type (0.1 M H_2O_2)	Survival Ratio as a Fold of Wild Type (0.2 M H_2O_2)
905	170,000,000	9,200	23	-	-
Final Population	26,000,000	16,000,000	7,000,000	11,371	1,989,967
B1	340,000	160,000	170,000	8,696	3,695,652
B3	46,000,000	16,000	35,000	6.427	5,624
B4	17,000,000	2,400,000	17,000	2,609	7,391
B6	9,200,000	2,400,000	5,400	4,820	4,338
B7	26,000,000	7,000,000	240	4,975	68
B8	17,000,000	1,600,000	1,100	1,739	478
B11	9,200,000	3,500,000	1,600	7,030	1,285
B12	9,200,000	3,500,000	160,000	7,030	128,544
B13	17,000,000	5,400,000	2,400	5,870	1,043
B14	35,000,000	3,500,000	16,000	1,848	3,379

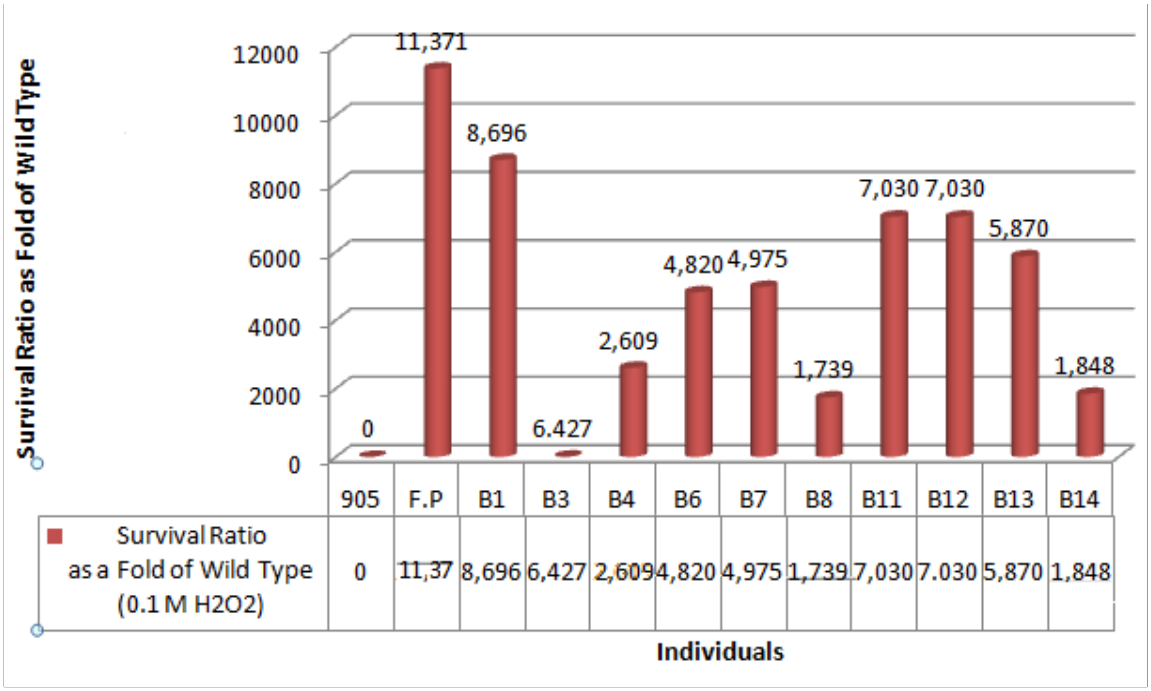


Figure 3.6 : Survival ratios of individuals as fold of wild type upon 0.1M H_2O_2 continuous stress (After 72 h)

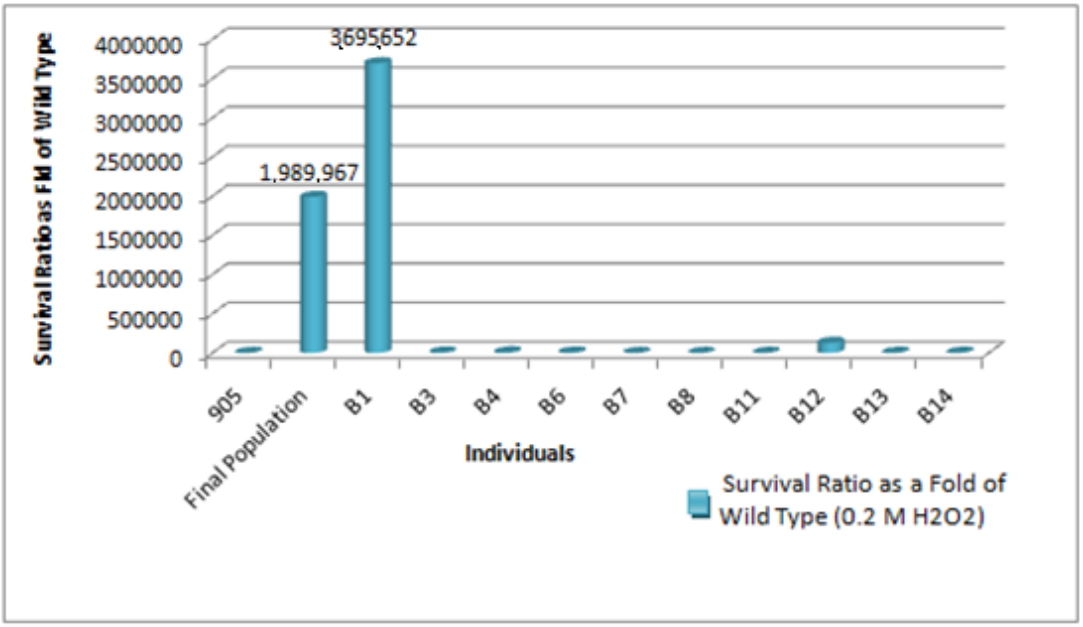


Figure 3.7 : Survival ratios of individuals as fold of wild type upon 0.2 M H_2O_2 continuous stress (After 72h)

3.2.2 Pulse stress resistance of individuals

Overnight liquid cultures of selected highly resistant individuals was diluted between A_{600} – 0.3 and inoculated at 150 rpm and 30°C till A_{600} 1,2 ; and then 50 mM H_2O_2 was applied during 90 minute at 150 rpm and 30°C.At the end of 90 minute individuals were washed twice with YMM then 20 µl sample from each individuals put into the wells that contain180 µl YMM. In each step 20 µl sample will mix with YMM and transfer the next row repeatedly until the final (8^{th}) row. Culture were inoculated at 30°C for 72 hour and during three days period visible growth of the each well noted. The results for 72^{th} hour of incubation are given in Table 3.5.

Table 3. 5 : Survival ratios of mutant individuals and wild type upon 50 mM H_2O_2 pulse stress application (After 72 h).

Individuals	**Number of cells per ml (Control)**	**Number of cells per ml 50mM H_2O_2**	**Per-cent Survival Value**	**Survival Ratio as a Fold of Wild Type (0.1 M H_2O_2)**
905	920,000	2,400	0.261	-
Final Population	540,000	11,000,000	2,037	7,809
B1	17,000	460,000	2,706	10,373
B3	920,000	240,000	26	100
B4	540,000	920,000	170	653
B6	540,000	540,000	100	383
B7	350,000	540,000	154	591
B8	1,100,000	110,000	10	38
B11	160,000	1,800,000	1,125	4,313
B12	1,700,000	1,600,000	94	361
B13	2,400,000	1,700,000	71	272
B14	170,000	350,000	206	789

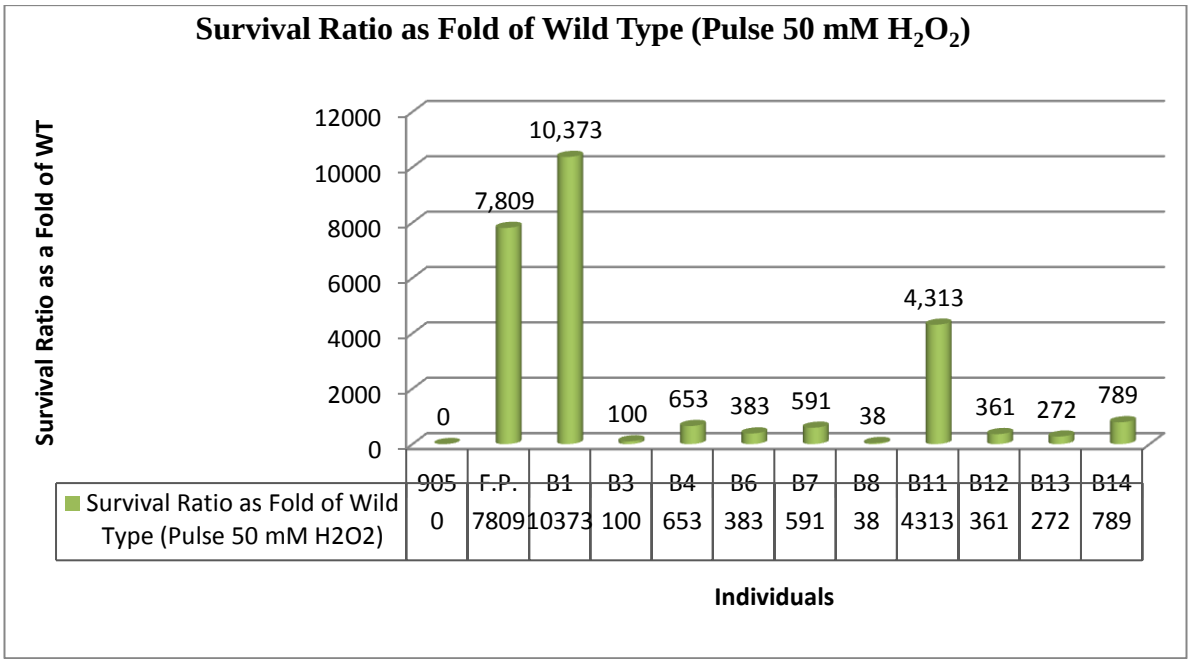


Figure 3.8 : Survival ratios of individuals as fold of wild type upon 50 mM H_2O_2 pulse stress (After 72 h)

3.3 Analysis of cross-stress resistances of mutant individuals on agar plate

Overnight liquid cultures of selected highly resistant individuals was diluted between A_{600} 0.2– 0.3 and inoculated at 150 rpm and 30^{o}C till A_{600} 1.2, then 5 micro liters of all samples were inoculated into YMM plates containing 2 mM sorbitol (w/v), 4 % NaCl (v/v), 0.25 mM $CuCl_2$, 2 mM $FeCI_2$, 2 mM $CrCl_3$, 2 mM $CoCl_2$, and 7% (v/v) ethanol respectively. The results of wild type and individuals without any stress conditions for 72th hour of incubation are given in Figure 3.9 and that group is used as control.

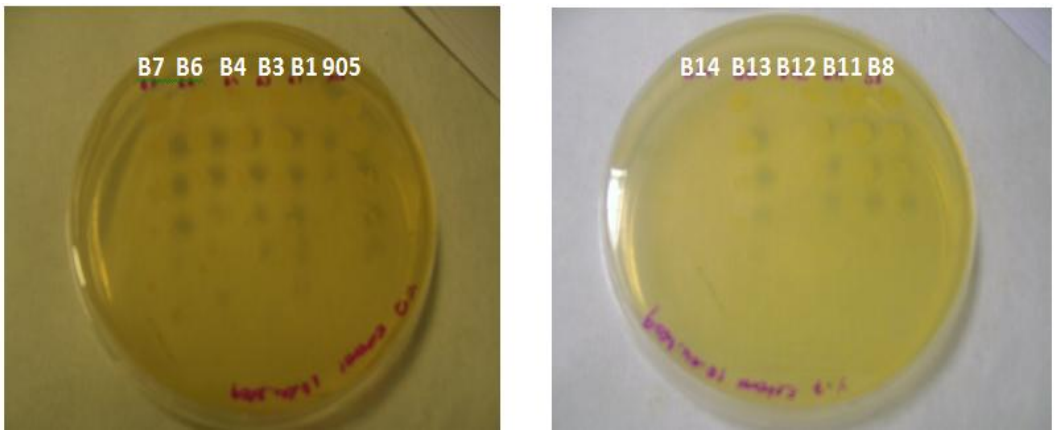


Figure 3.9 : The Growth of wild type and selected individuals on YMM plate without stress at 72th hour of incubation. (Columns up to bottom: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted)

3.3.1 Analysis of ethanol stress resistance on solid medium

Five micro liters of overnight cultures of mutant individuals B1, B3, B4, B6, B8, B11, B12, B13 and B14 and wild type 905 were inoculated into YMM plates which containing 7 % ethanol v/v. The growth was showed for 905 and all individuals on agar plate for 24, 48 and 72th hours of incubation.

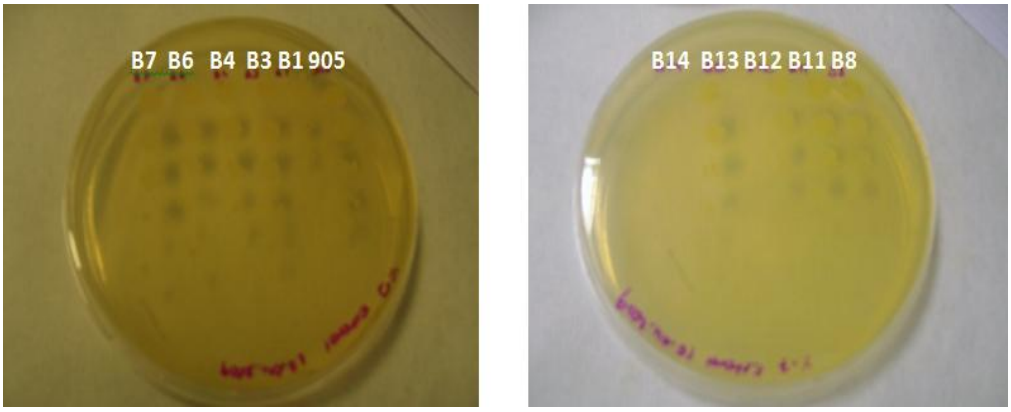


Figure 3.10 : The Growth of wild type and selected individuals on YMM plate with 7 % ethanol stress at 72th hour of incubation.(Columns up to bottom: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted)

3.3.2 Analysis of CuCl2 stress resistance on solid medium

Five micro liters of overnight cultures of mutant individuals B1, B3, B4, B6, B8, B11, B12, B13 and B14 and wild type 905 were inoculated into YMM plates containing 0.25 mM $CuCI_2$; the growth was showed for 905 and all individuals on agar plate for 24, 48 and 72th hours of incubation.

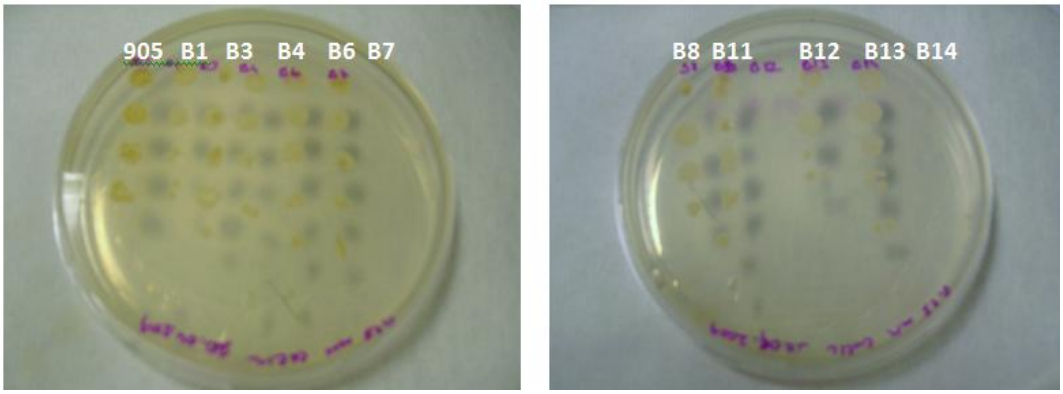


Figure 3.11 : The Growth of wild type and selected individuals on YMM plate with 0.25 mM $CuCI_2$ stress at 72th hour of incubation.(Columns up to bottom: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted).

3.3.3 Analysis of $CrCl_3$ stress resistance on solid medium

Five micro liters of overnight cultures of mutant individuals B1, B3, B4, B6, B8, B11, B12, B13 and B14 and wild type 905 were inoculated into YMM plates containing 2 mM $CrCI_3$; the growth was showed for 905 and all individuals on agar plate for 72^{th} hours of incubation.

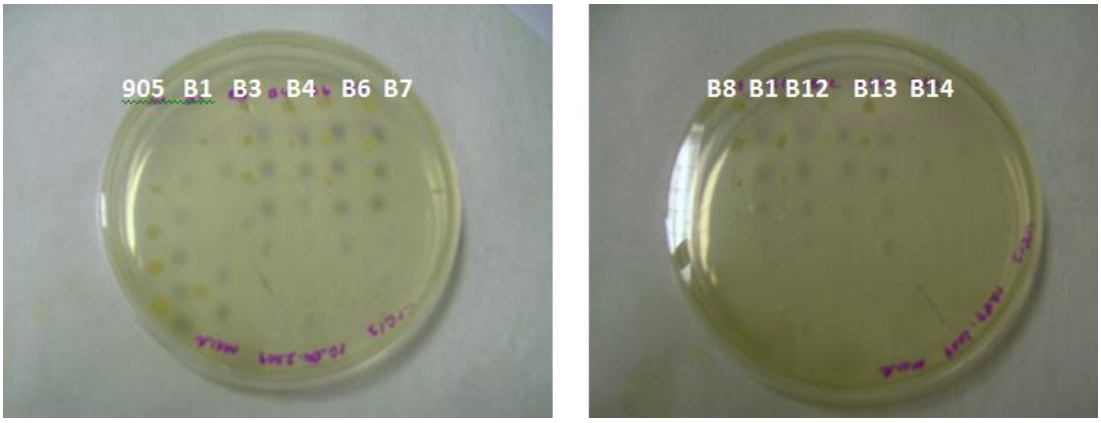


Figure 3.12 : The Growth of wild type and selected individuals on YMM plate with 2 mM $CrCI_3$ stress at 72^{th} hour of incubation.(Columns up to bottom: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted).

3.3.4 Analysis of $FeCl_2$ stress resistance on solid medium

Five micro liters of overnight cultures of mutant individuals B1, B3, B4, B6, B8, B11, B12, B13 and B14 and wild type 905 were inoculated into YMM plates containing 2 mM $FeCl_2$; the growth was showed for 905 and all individuals on agar plate for 72^{th} hours of incubation.

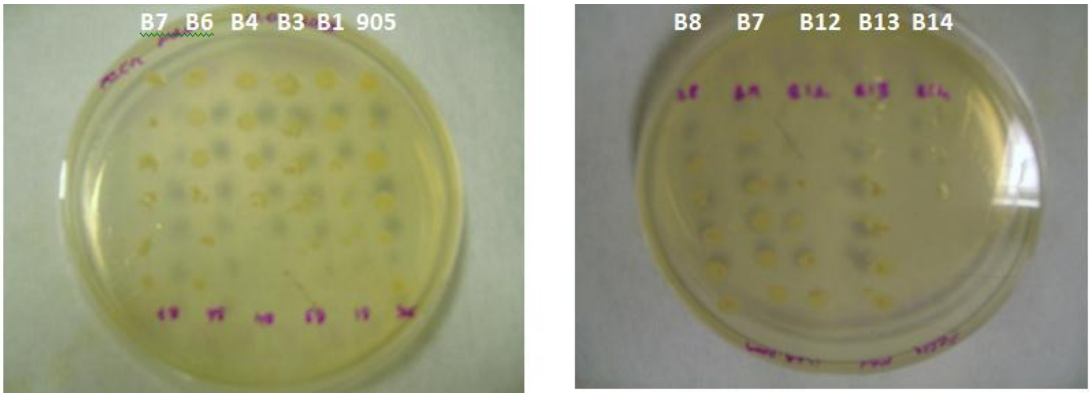


Figure 3. 13 : The Growth of wild type and selected individuals on YMM plate with 2 mM $FeCI_2$ stress at 72^{th} hour of incubation.(Columns up to bottom: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted).

3.3.5 Analysis of $CoCl_2$ stress resistance on solid medium

Five micro liters of overnight cultures of mutant individuals B1, B3, B4, B6, B8, B11, B12, B13 and B14 and wild type 905 were inoculated into YMM plates containing 2 mM $CoCl_2$; the growth was showed for 905 and all individuals on agar plate for 72^{th} hours of incubation

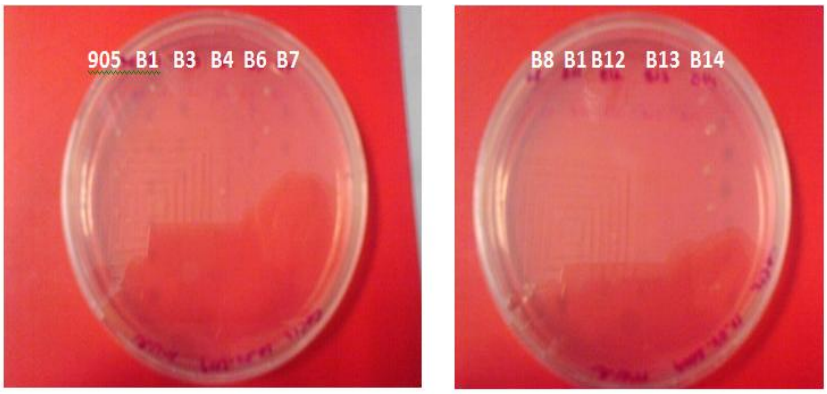


Figure 3.14 : The Growth of wild type and selected individuals on YMM plate with 2 mM $CoCI_2$; stress at 72^{th} hour of incubation.(Columns up to bottom: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted).

3.3.6 Analysis of osmotic stress resistance on solid medium

Five micro liters of overnight cultures of mutant individuals B1, B3, B4, B6, B8, B11, B12, B13 and B14 and wild type 905 were inoculated into YMM plates containing 2 mM sorbitol (w/v); 6 % NaCl (v/v), 4 % NaCl (v/v), but there was no growth was observed for 905 and all individuals on agar plate for 72th hours of incubation. The results were shown on Figure 3.15, Figure 3.16 and Figure 3.17 for 2 mM sorbitol (w/v), 4 % NaCl (v/v) and 6% NaCl (v/v) respectively.

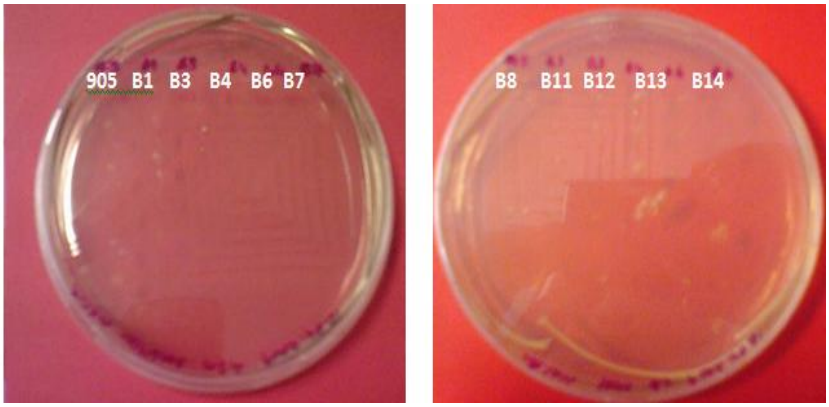


Figure 3.15 : The Growth of wild type and selected individuals on YMM plate with 2 mM Sorbitol; stress at 72^{th} hour of incubation.(Columns up to bottom: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted).

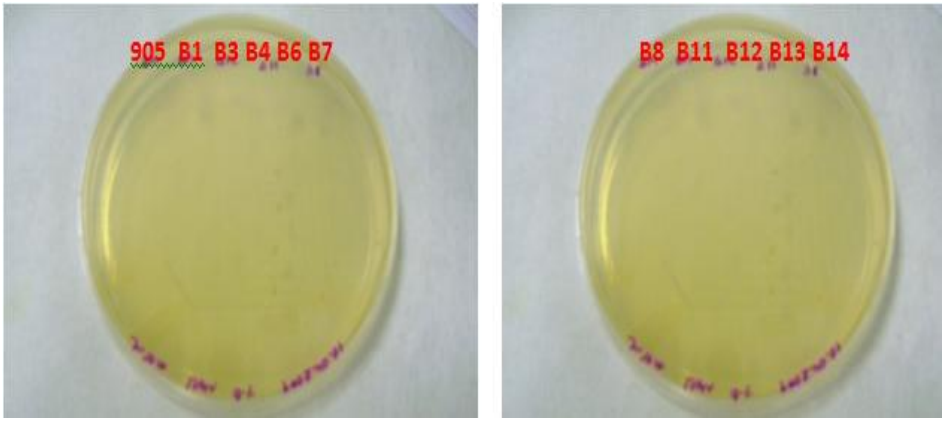


Figure 3.16 : The Growth of wild type and selected individuals on YMM plate with 6 % NaCl (v/v), stress at 72th hour of incubation. (Columns up to bottom: 10^{0}, 10^{-1},10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted).

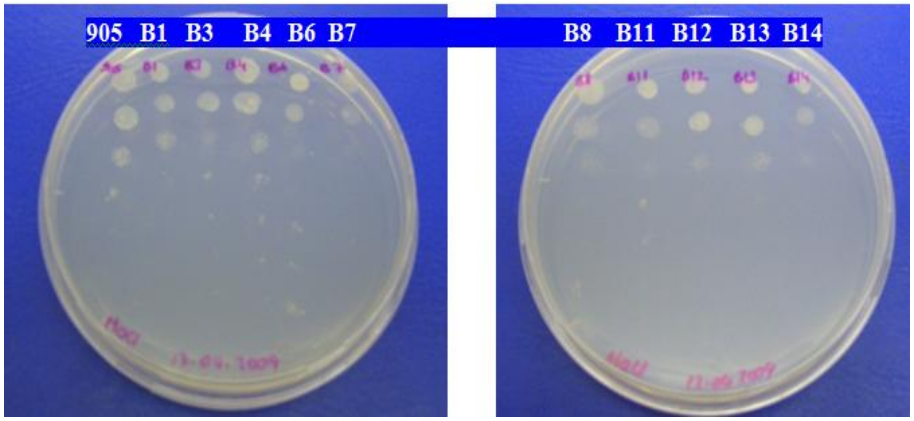


Figure 3.17 : The Growth of wild type and selected individuals on YMM plate with 4 % NaCl (v/v), stress at 72th hour of incubation.(Columns up to bottom: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} diluted).

3.4 Determination cross stress resistance of individuals with MPN

3.4.1 Heat stress

Wild type 905 and all mutants (B1, B3, B4, B6, B7, B8, B11, B12, B13 and B14) were cultured overnight and then for each sample 1 ml of overnight cultures was taken and washed with dextrose-free YMM and exposed to 60^{0} C temperature stress for 10 min. After this period of time the number of cells per ml and the percent survival values were determined by 5 tube- MPN method. The cultures with 30 ^{0}C exposure were used as control groups. The results at 72th hour of incubation are given in Table 3.6.

Table 3.6 : Survival ratios of selected mutant individuals and the wild type upon pulse heat stress (60°C, 10min) (After 72h)

Individuals	Number of cells per ml (Control)	Number of cells per ml (-60 ^{0}C)	Percent Survival Ratio at 60°C	Survival As Fold of Wild Type (at 60°C)
905	9,200,000	9,200	0.10	-
B1	7,000,000	17,000	0.24	2.43
B3	9,200,000	240,000	2.61	26.09
B4	1,600,000	11,000	0.69	6.88
B6	3,500,000	23	0.00	0.01
B7	9,200,000	3,500	0.04	0.38
B8	54,000,000	33	0.00	0.00
B11	17,000,000	16,000	0.09	0.94
B12	3,500,000	23	0.00	0.01
B13	1,700,000	24,000	1.41	14.12
B14	2,400,000	11	0.00	0.00

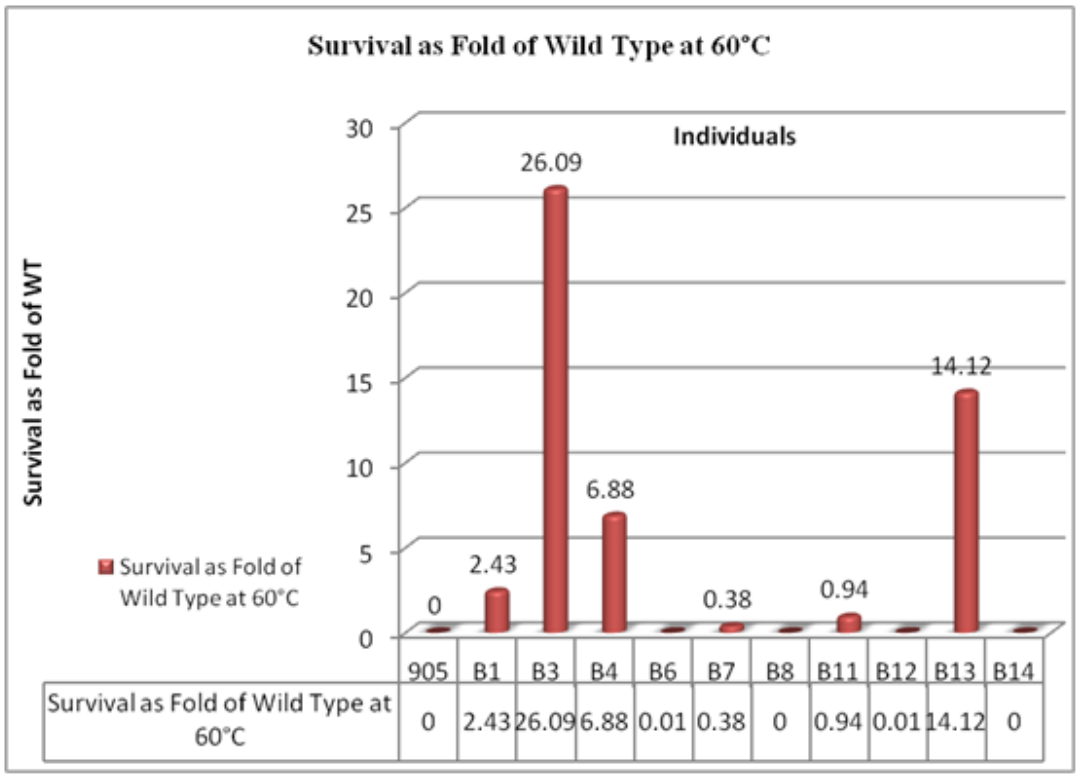


Figure 3.18 : Percent Survival ratios of individuals as fold of wild type upon 60^{o}C heat pulse stress (After 72 h)

3.4.2 Freeze-Thaw Stress

Wild type 905 and all mutants (B1, B3, B4, B6, B7, B8, B11, B12, B13 and B14) were cultured overnight and then for each sample 1 ml of overnight cultures were taken and washed with dextrose-free YMM and exposed to -20^{0}C and -196^{0}C freeze thaw stress separately for 90 minutes and 10 minutes respectively. After this period of time, all survivals were determined by 5 tube-MPN method. The number of cells per ml and the percent survival values were determined by 5 tube- MPN methods. The cultures with 30 ^{0}C exposure were used as control groups. The results at 72th hours of incubation are given in Table3.7 for -20^{0}C and -196^{0}C.

Table 3.7 : Survival ratios of selected mutant individuals and the wild type. (-20°C, 90min) (After 72h)

Individuals	Number of cells per ml (Control)	Number of cells per ml (-20 0C)	Percent Survival Ratio at -20°C	Survival As Fold of Wild Type (at -20°C)	Number of cells per ml (-196^{0}C)	Percent Survival Ratio at -196°C	Survival As Fold of Wild Type (at -196°C)
905	9,200,000	16,000,000	174	-	110,000	1.20	-
B1	7,000,000	2,400,000	34	0.20	540	0.01	0.01
B3	5,400,000	35,000,000	648	3.73	340	0.00	0.00
B4	1,600,000	9,200,000	575	3.31	9,200	0.58	0.48
B6	3,500,000	9,200,000	263	1.51	9,200	0.26	0.22
B7	9,200,000	35,000,000	380	2.19	920,000	10.00	8.36
B8	54,000,000	23	0	0.00	4,600	0.01	0.01
B11	17,000,000	7,000,000	41	0.24	46,000	0.27	0.23
B12	3,500,000	2,400,000	68	0.39	17,000	0.49	0.41
B13	1,700,000	540,000	31	0.18	22,000	1.29	1.08
B14	2,400,000	9,200,000	383	2.20	920,000	38.33	32.06

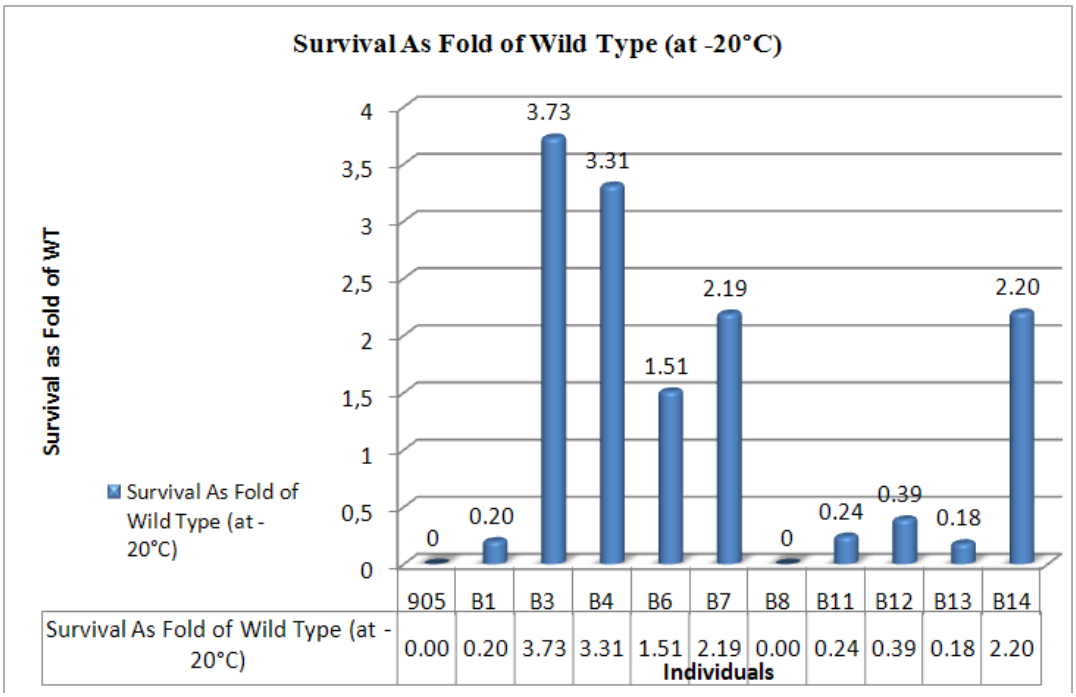


Figure 3. 19 : Survival ratios of selected mutant individuals and the wild type.(-20°C, 90min) (After 72h)

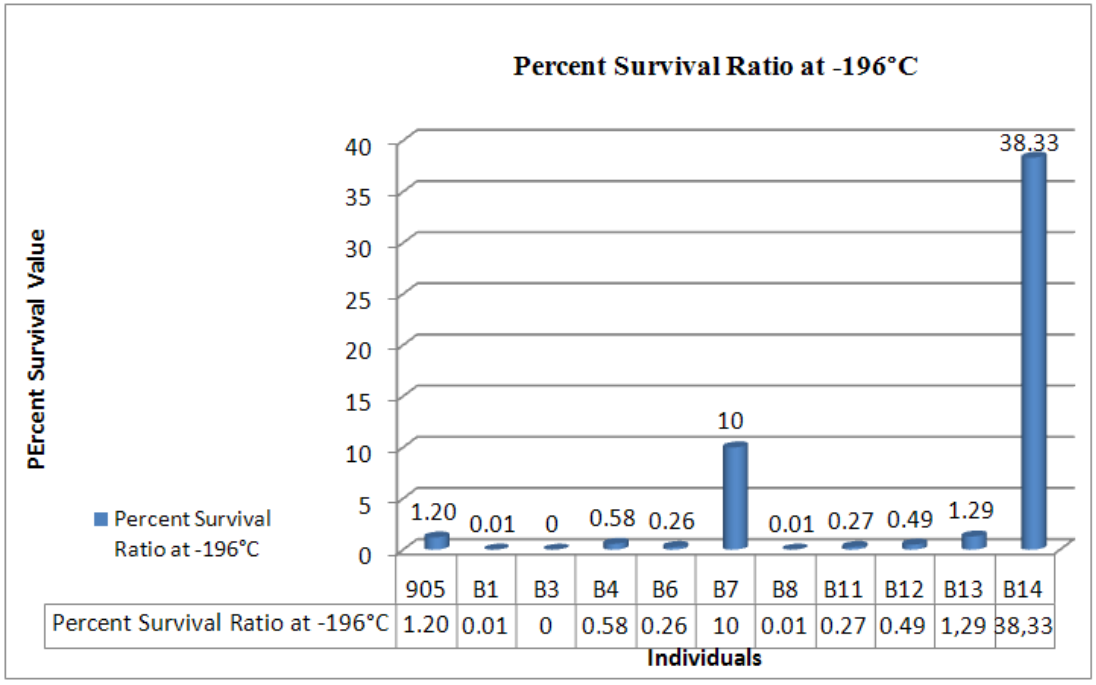


Figure 3.20: Survival ratios of selected mutant individuals and the wild type. (-196°C, 10min) (After 72h)

3.4.3 Iron Stress

Wild type 905 and all mutants (B1, B3, B4, B6, B7, B8, B11, B12, B13 and B14) were cultured overnight and then the samples were diluted in appropriate ratio and incubated at 30°C, 150 rpm till reached OD_{600} value of 1.2. In this step 1 ml of each sample was taken and washed with dextrose-free YMM and exposed to 2 mM $FeCl_2$ and 5 mM $FeCl_2$ for 90 min. At the end of time period, all samples were centrifuged for 5 min. at 14,000 rpm and supernatant was discarded. All samples were washed twice with YMM without dextrose and centrifuged again for 5 min. at 14,000 rpm. The number of cells per ml and the percent survival values were determined by 5 tube- MPN methods. The results at 72^{th} hours of incubation are given in Table 3.8 for 2 mM $FeCl_2$ and 5 mM $FeCl_2$

Table 3. 8 : Survival ratios of selected mutant individuals and the wild type that exposed to 2 mM $FeCI_2$ and 5 mM $FeCI_2$ for 90 minute (After 72h)

Individuals	Number of cells per ml (Control)	Number of cells per ml at 2 mM $FeCl_2$	Percent Survival Ratio at - at 2 mM $FeCl_2$	Survival As Fold of Wild Type at 2 mM $FeCl_2$	Number of cells per ml at 5 mM $FeCl_2$	Percent Survival Ratio at 5 mM $FeCl_2$	Survival As Fold of Wild Type at 5 mM $FeCl_2$
905	2,400,000	1,600,000	67	-	3,500,000	146	-
Final Population	1,700,000	1,600,000	94	1.41	3,500,000	206	1.41
B1	1,700,000	1,700,000	100	1.50	920,000	54	0.37
B3	2,400,000	9,200,000	383	5.75	3,500,000	146	1
B4	1,700,000	2,200,000	129	1.94	1,100,000	65	0.44
B6	1,600,000	5,400,000	338	5.06	3,500,000	219	1.50
B7	1,600,000	1,800,000	113	1.69	350,000	22	0.15
B8	2,200,000	2,200,000	100	1.50	14,000,000	636	4.36
B11	240,000	3,500,000	1,458	21.88	1,600,000	667	4.57
B12	3,500,000	35,000,000	1,000	15.00	7,000,000	200	1.37
B13	540,000	350,000	648	9.72	1,600,000	296	2.03
B14	920,000	7,000,000	761	11.41	2,400,000	261	1.79

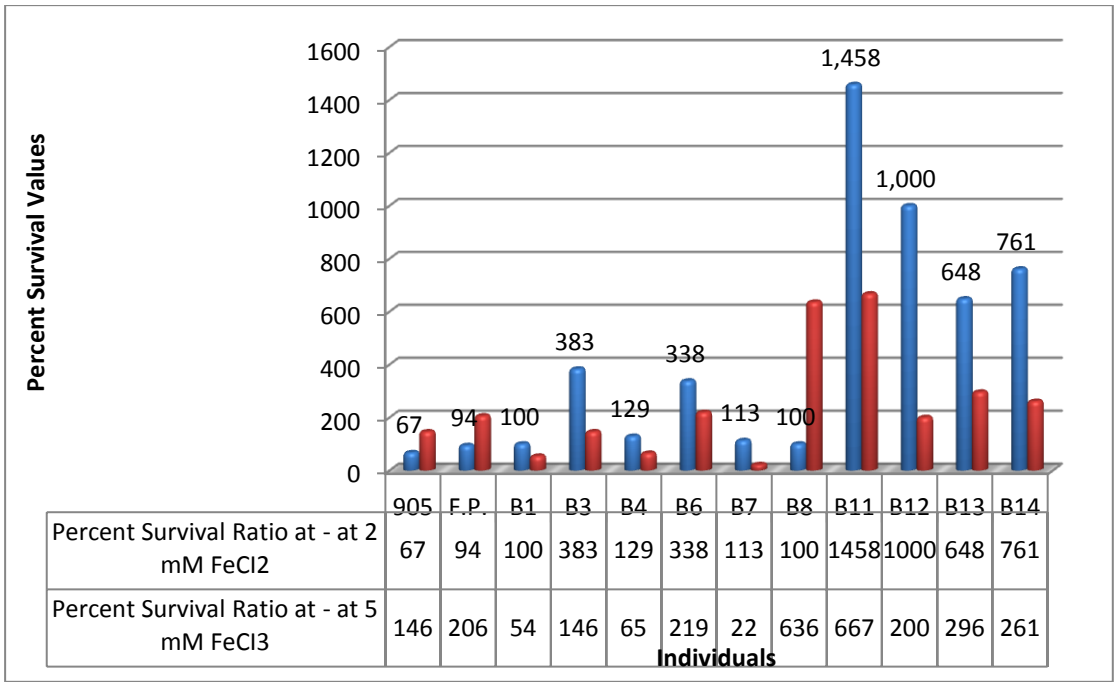


Figure 3. 21 : Percent survival ratios of selected mutant individuals and the wild type that exposed 2 mM $FeCI_2$ and 5 mM $FeCI_2$ for 90 minute. (After 72h)

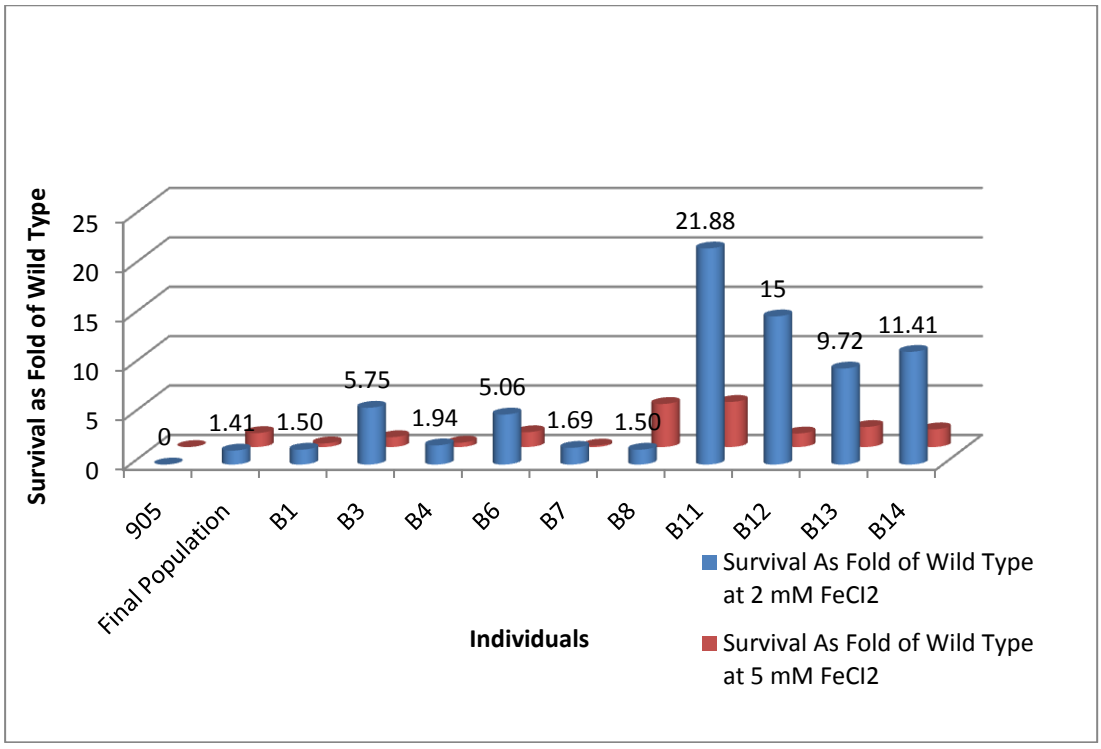


Figure 3.22: Survival ratios of selected mutant individuals and the wild type that exposed 2 mM $FeCI_2$ and 5 mM $FeCI_2$ for 90 minute. (After 72h)

3.4.4 Ethanol Stress

Wild type 905 and all mutants (B1, B3, B4, B6, B7, B8, B11, B12, B13 and B14) were cultured overnight and then the samples were diluted in appropriate ratio and incubated at 30°C, 150 rpm till reached OD_{600} value of 1.2. In this step 1 ml of each sample was taken and washed with dextrose-free YMM and exposed to 5 % ethanol and 7 % ethanol for 90 minute. At the end of time period, all samples were centrifuged for 5 minute at 14.000 rpm and supernatant was discarded. All samples were washed twice with YMM without dextrose and centrifuged again for 5 minute at 14.000 rpm. The number of cells per ml and the percent survival values were determined by 5 tube- MPN methods. The results at 72^{th} hours of incubation are given in Table3.9 for 5 % and %8 (v/v) ethanol.

Table 3.9 : Survival ratios of selected mutant individuals and the wild type that exposed to 5 % and 7% ethanol for 90 minute (After 72h).

Individuals	Number of cells per ml (Control)	Number of cells per ml 5 % ethanol	Percent Survival Ratio at -5 % ethanol	Survival As Fold of Wild Type at 5 % ethanol	Number of cells per ml 8 % ethanol	Percent Survival Ratio at 8 % ethanol	Survival As Fold of Wild Type at 8 % ethanol
905	16,000,000	9,200,000	57.5	-	9,200,000	58	-
Final Population	3,500,000	5,400,000	154.3	2.68	5,400,000	154	2.68
B1	5,400,000	1,700,000	31.5	0.55	3,500,000	65	1.13
B3	1,700,000	1,600,000	94.1	1.64	3500	0	0
B4	3,500,000	920,000	26.3	0.46	1,600,000	46	0.01
B6	920,000	2,400,000	260.9	4.54	3,500,000	380	6.56
B7	5,400,000	1,000,000	18.5	0.32	54,000	1	0.02
B8	54,000,000	1,700,000	3.1	0.05	920,000	2	0.03
B11	3,500,000	1,600,000	45.7	0.80	1,600,000	46	0.80
B12	52,000,000	5,400,000	10.4	0.18	16,000,000	31	0.54
B13	2,400,000	1,700,000	70.8	1.23	920,000	38	0.67
B14	9,200,000	3,500,000	38.0	0.66	2,200,000	24	0.42

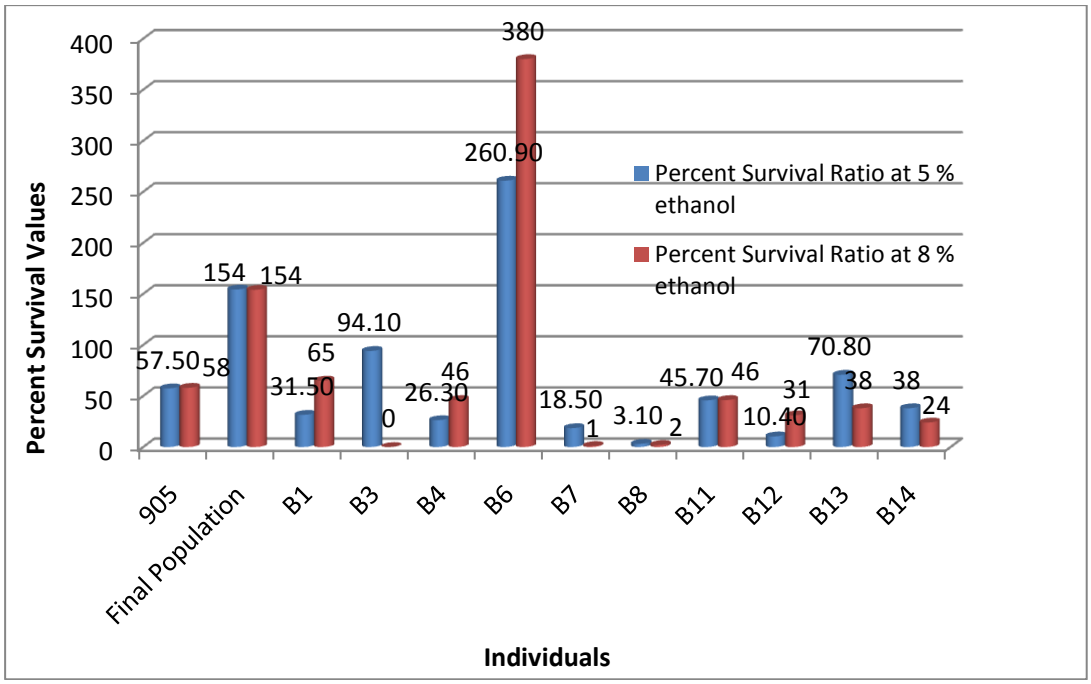


Figure 3.23 : Percent survival ratios of selected mutant individuals and the wild type that exposed 5 % and 7 % ethanol for 90 minute. (After 72h),

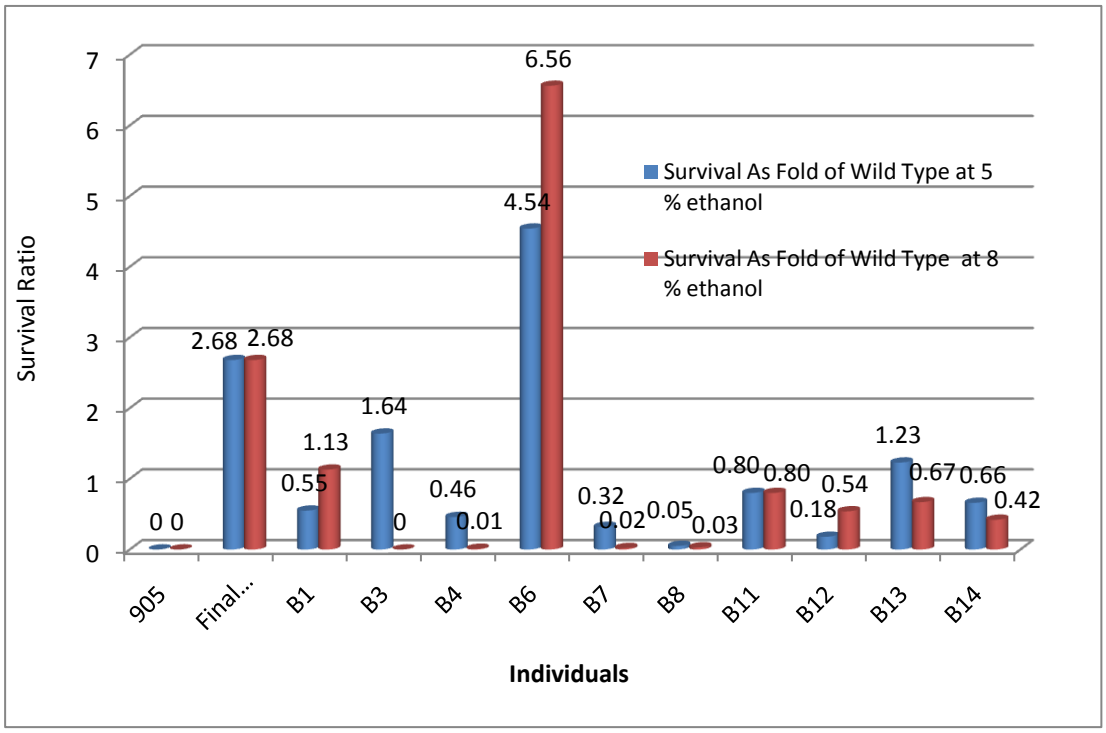


Figure 3.24 : Survival ratios of selected mutant individuals and the wild type that exposed 5 % and 7 % ethanol for 90 minute. (After 72h)

3.4.5 Copper Stress

Wild type 905 and all mutants (B1, B3, B4, B6, B7, B8, B11, B12, B13 and B14) were cultured overnight and then the samples were diluted in appropriate ratio and incubated at 30°C, 150 rpm till reached OD_{600} value of 1.2. In this step 1 ml of each sample was taken and washed with dextrose-free YMM and exposed to 0.25 mM $CuCI_2$ for 90 min. At the end of time period, all samples were centrifuged for 5 min. at 14,000 rpm and supernatant was discarded. All samples were washed twice with YMM without dextrose and centrifuged again for 5 min. at 14,000 rpm. The number of cells per ml and the percent survival values were determined by 5 tube- MPN methods. The results at 72^{th} hours of incubation are given in Table 3.10.

Table 3.10 : Survival ratios of selected mutant individuals and the wild type that exposed to 0.25 mM $CuCl_2$ for 90 minute (After 72h)

Individuals	Number of cells per ml (Control)	Number of cells per ml 0.25 mM $CuCI_2$	Percent Survival Ratio at 0.25 mM CuCI2	Survival As Fold of Wild Type at 0.25 mM CuCI2
905	2,400,000	5	0.00	-
Final Population	1,700,000	23	0.00	6.49
B1	1,700,000	0.01	0.00	0.00
B3	2,400,000	8	0.00	1.60
B4	1,700,000	0.01	0.00	0.00
B6	1,600,000	2	0.00	0.60
B7	1,600,000	0.01	0.00	0.00
B8	2,200,000	23	0.00	5.02
B11	240,000	0.01	0.00	0.02
B12	3,500,000	5	0.00	0.69
B13	540,000	23	0.00	20.44
B14	920,000	23	0.00	12.00

3.5 Growth curve analysis

3.5.1 Spectrophotometric analysis of growth

In this part of the study, growth physiology of the wild type and the mutant individual with highest resistance, B1, B11 and B14 was determined in the presence and absence of 1 mM H2O2 in the culture medium containing YMM. After sampling at 0^{th}, 3^{th}, 6^{th}, 12^{th}, 18th,21th, 24^{th}, 27^{th}, 30^{th} ,33^{th} and 45^{th} hours of incubation, the measured OD600 values of wild type (905) and B1,B11 and B14 are shown in Table 3.11.

Table 3.4 : OD_{600} values of 905 and individuals (B1, B11 and B14) depend on time. The upper table shows control groups and below shows 1 mM H2O2 stress exposed group.

	Time Point-0	3 th Hour	6th Hour	9th Hour	12 th Hour	18 th Hour	21 th Hour	4 th Hour	27 th Hour	30 th Hour	36 th Hour	45 th Hour
905C	0.05	0.255	0.537	1.11	4.692	5.88	5.25	5.18	5.04	5.322	5.684	5.413
B1C	0.05	0.093	0.424	0.79	2.328	4.6	4.64	4.89	4.86	4.423	6.234	6.135
B11C	0.05	0.104	0.383	0.82	2.91	5.85	4.97	5.37	5.69	6.010	6.930	6.565
B14C	0.05	0.123	0.46	0.81	3.162	5.54	5.2	4.81	6.2	6.578	8.132	7.959

905S	0.15		0.224	0.34	0.49	0.68	0.74	0.77	1.31	2.600	4.560	3.950
B1S	0.31		0.609	1.36	5.856	5.93	5.64	5.77	5.58	6.500	7.097	7.640
B11S	0.31		0.569	1.26	4.038	5.25	5.38	5.57	4.09	6.454	6.542	6.245
B14S	0.31		0.88	1.72	5.01	6.88	6.9	7.23	6.64	8.070	7.600	6.946

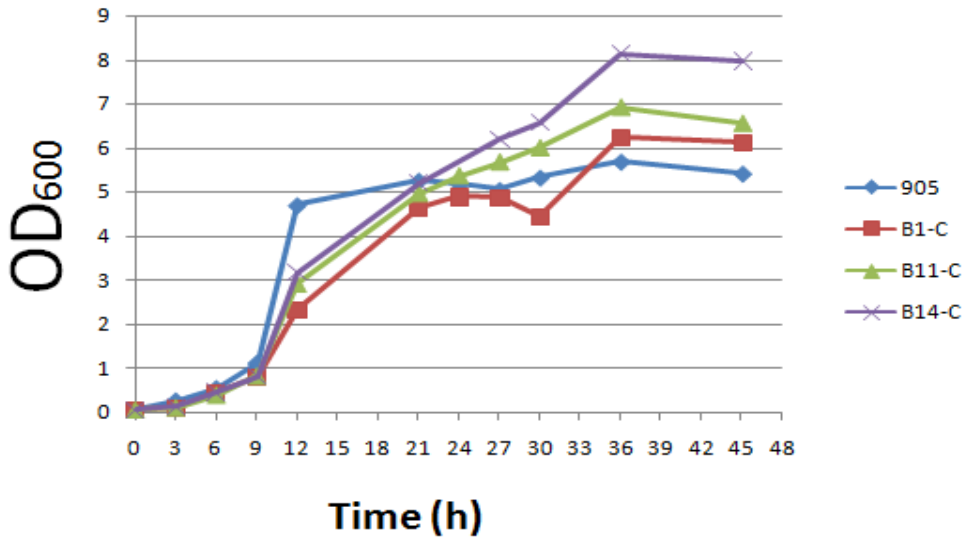


Figure 3.25 : Growth of wild type and all individual in the absence of 1mM H_2O_2 continuous stress conditions depends on OD_{600} measurements.

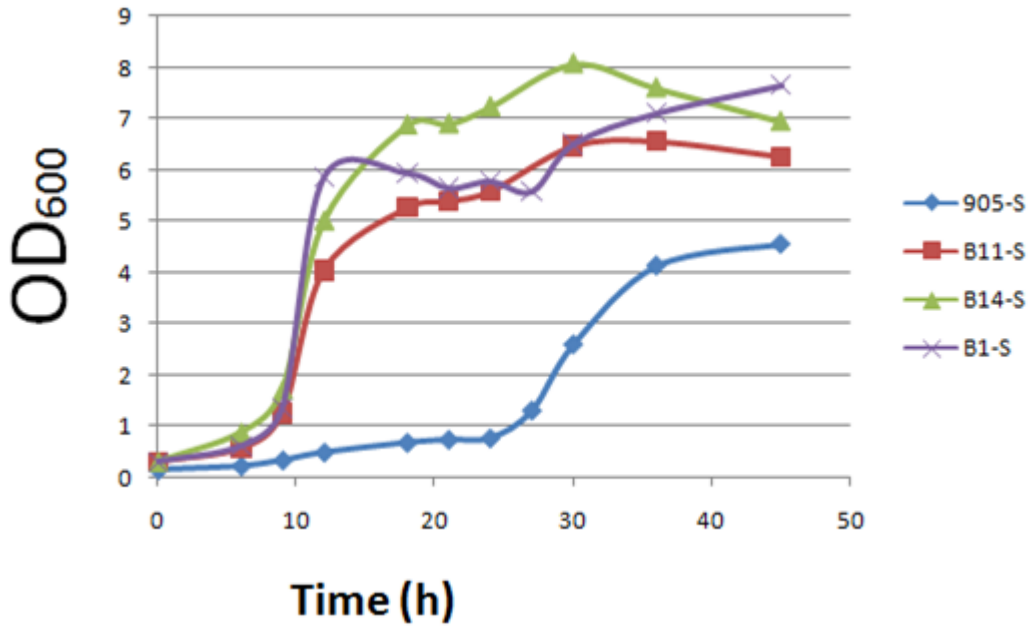


Figure 3.26 : Growth of wild type and all individual in the presence of 1mM H_2O_2 continuous stress conditions depends on OD600 measurements.

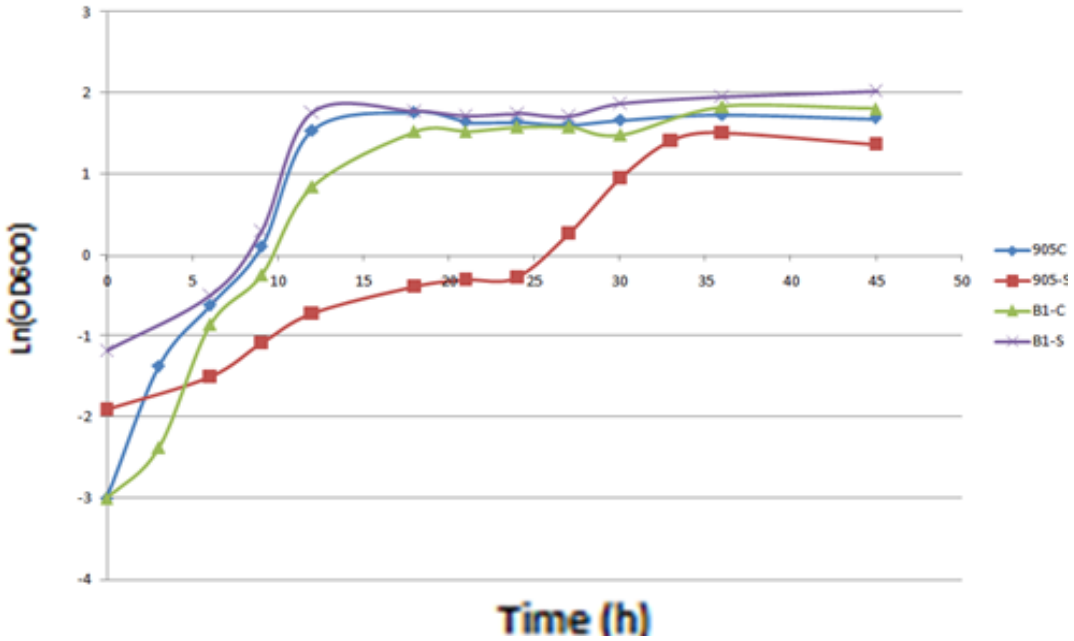


Figure 3.27 : Growth curve of wild type and B1 individual in the absence and presence of 1mM H_2O_2 continuous stress conditions, based on OD_{600} measurements

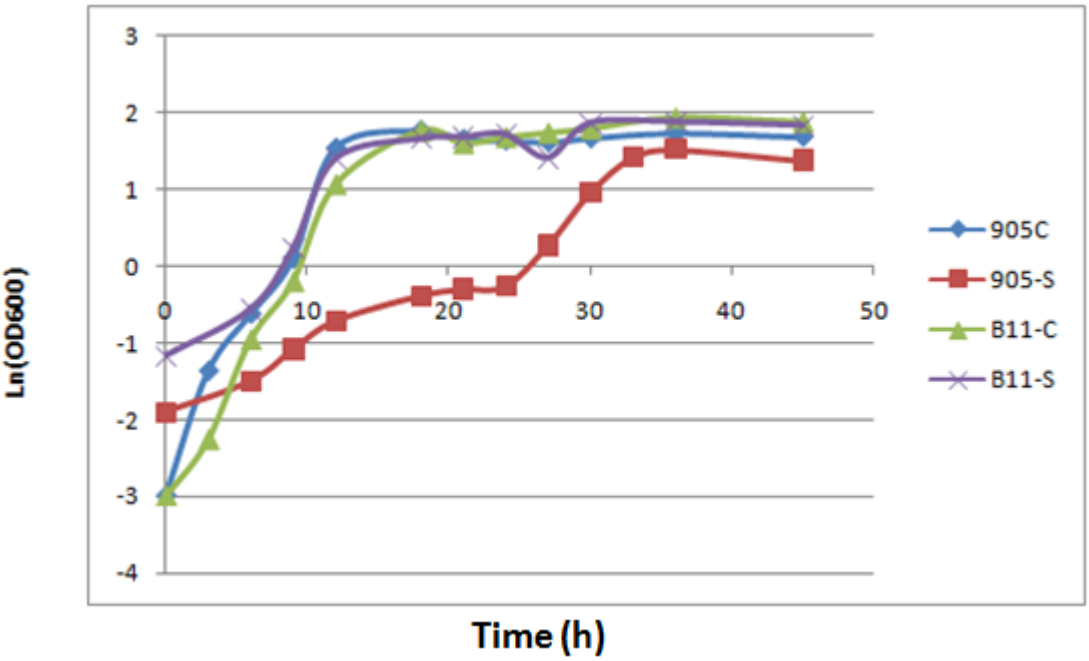


Figure 3.28 : Growth curve of wild type and B11 individual in the absence and presence of 1mM H_2O_2 continuous stress conditions, based on OD_{600} measurements

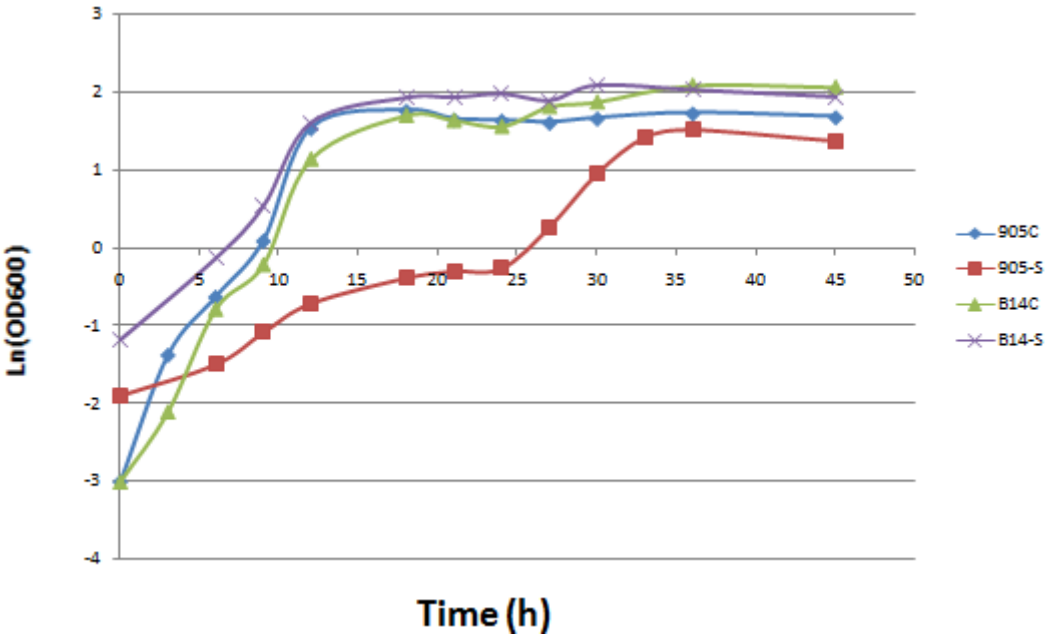


Figure 3. 29: Growth curve of wild type and B14 individual in the absence and presence of 1mM H_2O_2 continuous stress conditions, based on OD_{600} measurements

3.5.2 Catalase activity and Specific catalase activity measurement

Catalase activity measurement was performed spectrophotometrically by measuring the changes OD_{240} upon time. Bradford assay was used to determine total soluble protein amount. The enzyme activity is measured in conditions that guarantee substrate overflow,30^0C temperature and optimal pH. The catalase activity is expressed by quantity of H_2O_2 decomposed per time unit. Specific catalase activities were represented as ΔA240 /min/mg protein. The experiment was performed two times but the results were not correspond each other.

Table 3.52 : Total soluble protein concentrations (mg/ml) of the wild type and selected individuals in the absence and presence of 1 mM H_2O_2 continuous stress conditions.

Time (h)	905-C	905-S	B1-C	B1-S	B11-C	B11-S	B14-C	B14-S
12	1.226	0.125	0.377	0.051	0.558	0.044	0.398	0.044
18	0.975	0.125	0.470	0.300	0.392	0.400	0.491	0.250
24	2.636	2.518	3.449	3.817	3.817	3.962	4.439	3.758
27	0.844	0.257	1.065	0.240	1.030	0.308	1.323	0.880

Table 3.136 : A specific catalase activity (ΔA_{240} min/mg) of the wild type and selected individuals in the absence and presence of 1 mM H_2O_2 continuous stress conditions.

Time (h)	905-C	905-S	B1-C	B1-S	B11-C	B11-S	B14-C	B14-S
12	0.079	0.234	0.064	0.594	0.360	0.618	0.192	0.239
18	0.089	0.117	0.069	0.614	0.574	0.738	1.089	2.552
24	0.061	0.499	0.151	0.500	0.480	0.483	0.236	0.079
27	0.107	2.154	0.090	0.244	2.619	0.163	0.150	0.272

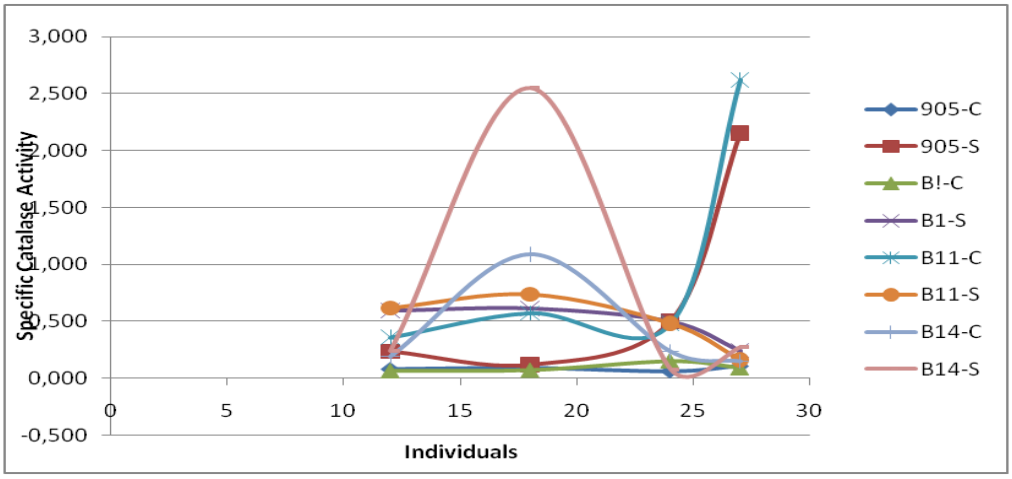


Figure 3.30 : Specific catalase activity ((ΔA_{240} min/mg) of the wild type and selected individuals in the absence and presence of 1 mM H_2O_2 continuous stress conditions.

3.6 Specific gene expression analyses using rt-PCR

3.6.1 RNA Isolation from wild type and individuals

Wild type and H_2O_2 exposed individuals "B1, B11 andB14" that obtained from pulse oxidative stress application were incubated in 1 lt flasks for 24 hours at 30^0C. During the growth, each sample has its own control flasks and stress flask that contain 1 mM H_2O_2. Total RNA isolation is controlled by 1.5 % agarose gel.

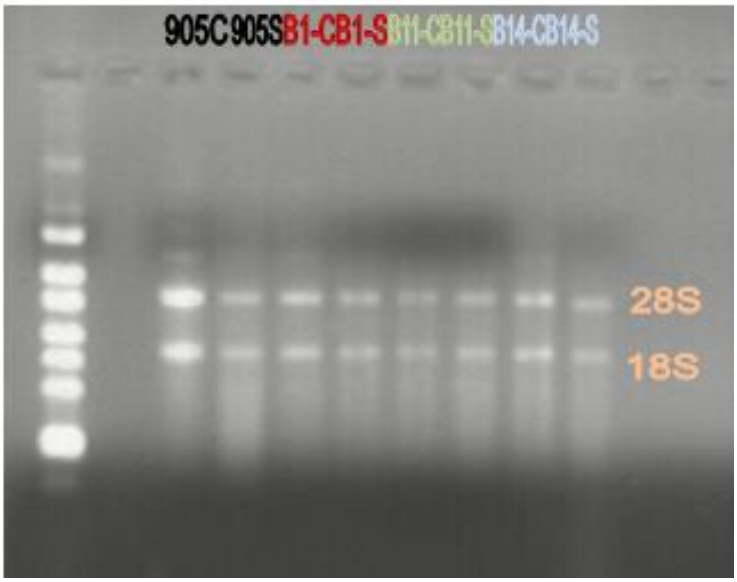


Figure 3.31 : RNA isolation of wild type and selected individuals. Fermantas Riboruler RNA Ladder High Range is used as marker. All samples were run on a 1.5% agarose gel.

3.6.2 Determination of Total RNA Concentrations

Total RNA concentrations of the samples were measured by Qubit Quantitation Platform. The RNA concentration that was determined by the machine is shown on Table 3.14.

Table 3.14 : Total RNA concentration of wild type and individuals as ng/ µl

	905-C	905-S	B1-C	B1-S	B11-C	B11-S	B14-C	B14-S
RNA Concentration	88.4	88.2	62.2	62.4	30.8	62.8	72.6	43.8

3.6.3 Cycle determination of relevant genes for rt-PCR

3.6.3.1 CTT1 cycle determination of selected individuals and wild type

The expression level of CTT1 was determined through RT-PCR by using ACT1 as internal control. RT-PCR application was done by using RNA samples of wild type and individual cultures which were isolated from cultures grown on YMM and 1 mM H_2O_2 containing YMM. According to their RNA concentrations, calculation was done and 100 ng RNA from each sample was used for RT-PCR studies. In order to determine at which cycle PCR product become saturated, PCR was lasted for 36 cycle and 10 µl sample was taken at 24^{nd}, 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle. These PCR products were loaded to agarose gel (1.5%).

Table 3.15 : RT-PCR cycle conditions for CTT1 gene

	Temperature (ºC)	**Time**	**Cycle number**
cDNA synthesis	55	30 min	1
Denaturation	94	2 min	1
PCR			
Denaturation	94	15 sec	**36**
Annealing	53	90 sec	
Extension	68	50 sec	
Final extension	68	5 min	1

PCR products taken at different PCR cycles were loaded to agarose gel and it was run for 45 min at 120 V. Then, the gel photograph was taken from the gel visualization system.

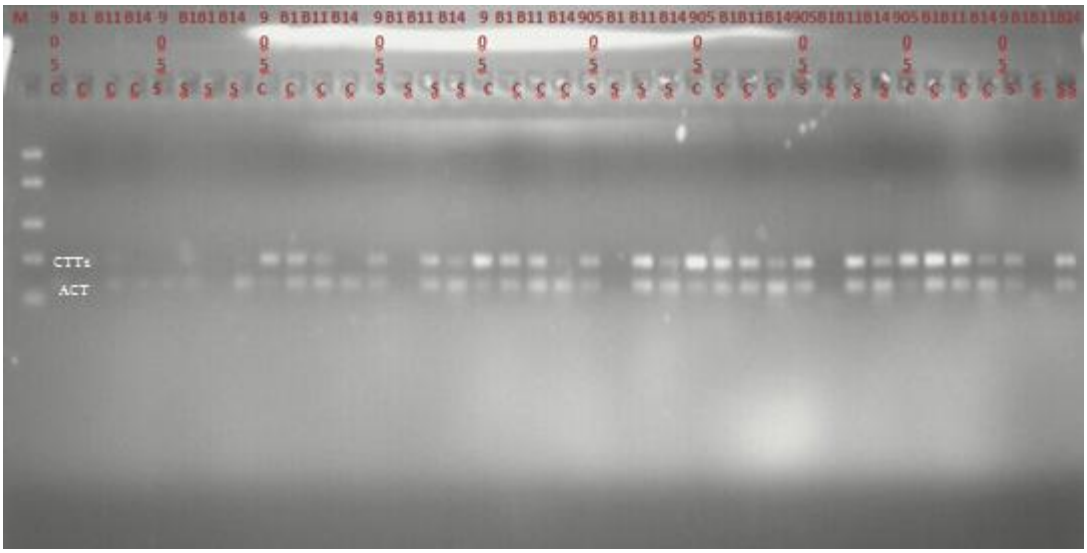


Figure 3.32 : RT-PCR experiment with CTT1 primer.Line1 Fermentas DNA Low Range DNA Ladder (#SM1103), individuals and theirs expression profile is shown on the figure above for 24^{nd}, 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle.

3.6.3.2 YAP1 cycle determination of selected individuals and wild type

The expression level of YAP1 was determined through RT-PCR by using ACT1 as internal control. RT-PCR application was done by using RNA samples of wild type and individuals which were isolated from cultures grown on YMM and 1 mM H2O2 containing YMM. According to their RNA concentrations, calculation was done and 100 ng RNA from each sample was used for RT-PCR studies. In order to determine at which cycle PCR product become saturated, PCR was lasted for 36 cycle and 10 µl sample was taken at 24nd, 27th, 30th, 33th, 36th cycle. These PCR products were loaded to agarose gel (1.5%).

Table 3.167 : RT-PCR cycle conditions for YAP1 gene

	Temperature (ºC)	**Time**	**Cycle number**
cDNA synthesis	55	30 min	1
Denaturation	94	2 min	1
PCR			
Denaturation	94	15 sec	**36**
Annealing	53	90 sec	
Extension	68	50 sec	
Final extension	68	5 min	1

PCR products taken at different PCR cycles were loaded to agarose gel and it was run for 30 min at 120 V. Then, the gel photograph was taken from the gel visualization system.

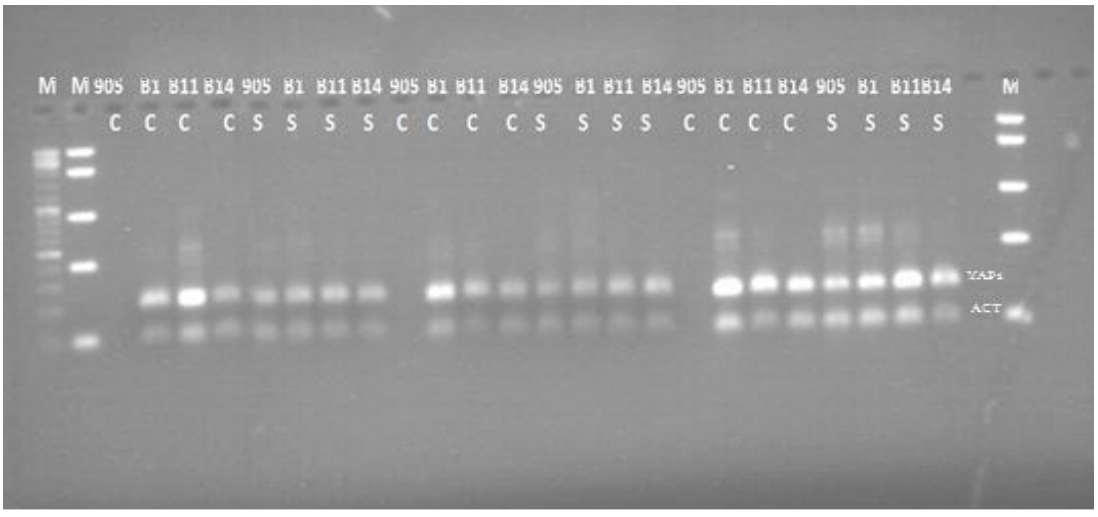


Figure 3.33 : RT-PCR experiment with YAP1 primer **Line 1** Fermentas Mass Ruler DNA Ladder, **Line 2 and 28** Fermentas Fast Ruler DNA Ladder Mix,individuals and theirs expression profile is shown on the figure above for 24nd, 27th, 30th, 33th, 36th cycle.

3.6.3.3 ATF1 cycle determination of selected individuals and wild type

The expression level of ATF1 was determined through RT-PCR by using ACT1 as internal control. RT-PCR application was done by using RNA samples of wild type and individuals which were isolated from cultures grown on YMM and 1 mM H2O2 containing YMM. According to their RNA concentrations, calculation was done and 100 ng RNA from each sample was used for RT-PCR studies. In order to determine at which cycle PCR product become saturated, PCR was lasted for 36 cycle and 10 µl sample was taken at 24^{nd}, 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle. These PCR products were loaded to agarose gel (1.5%).

Table 3.8 : RT-PCR cycle conditions for ATF1 gene.

	Temperature (ºC)	**Time**	**Cycle number**
cDNA synthesis	55	30 min	1
Denaturation	94	2 min	1
PCR			
Denaturation	94	15 sec	**36**
Annealing	53	90 sec	
Extension	68	50 sec	
Final extension	68	5 min	1

PCR products taken at different PCR cycles were loaded to agarose gel and it was run for 30 min at 120 V. Then, the gel photograph was taken from the gel visualization system.

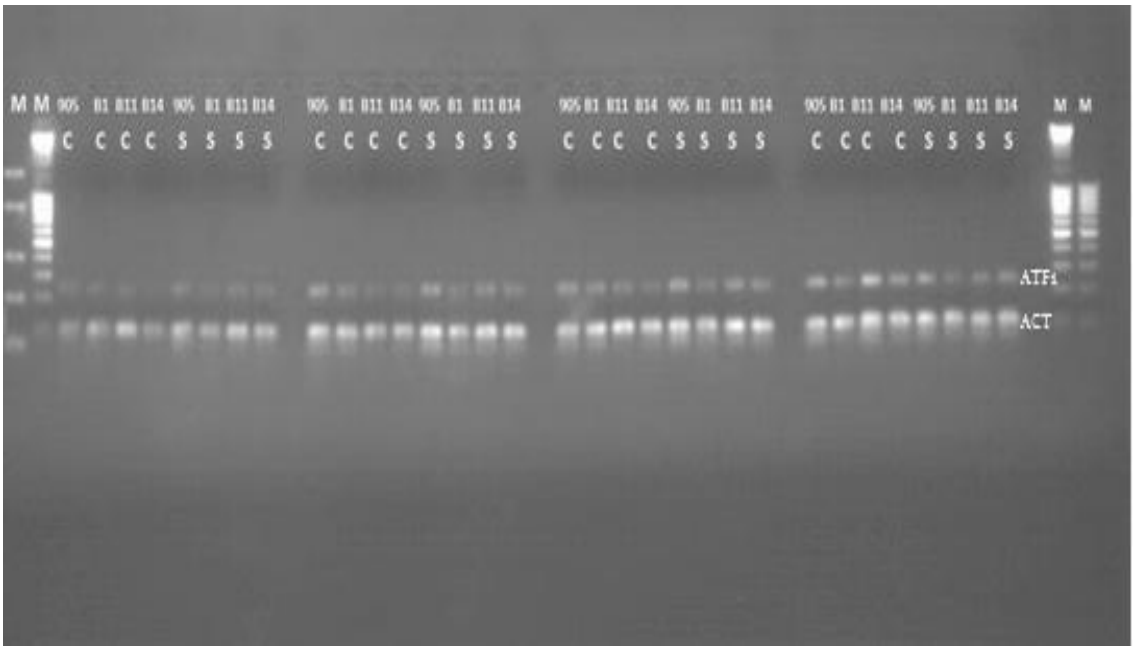


Figure 3.34 : RT-PCR experiment with ATF1 primer **Line 1** Fermentas Fast Ruler DNA Ladder Mix **Line 2 and 36** Fermentas Mass Ruler DNA Ladder, individuals and theirs expression profile is shown on the figure above for 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle.

3.6.3.4 GLR1 cycle determination of selected individuals and wild type

The expression level of GLR1 was determined through RT-PCR by using ACT1 as internal control. RT-PCR application was done by using RNA samples of wild type and individuals which were isolated from cultures grown on YMM and 1 mM H_2O_2 containing YMM. According to their RNA concentrations, calculation was done and 100 ng RNA from each sample was used for RT-PCR studies. In order to determine at which cycle PCR product become saturated, PCR was lasted for 36 cycle and 10 µl sample was taken at 24^{nd}, 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle. These PCR products were loaded to agarose gel (1.5%).

Table 3. 18 : RT-PCR cycle conditions for GLR1 gene

	Temperature (ºC)	**Time**	**Cycle number**
cDNA synthesis	55	30 min	1
Denaturation	94	2 min	1
PCR			
Denaturation	94	15 sec	**36**
Annealing	55	90 sec	
Extension	68	50 sec	
Final extension	68	5 min	1

PCR products taken at different PCR cycles were loaded to agarose gel and it was run for 30 min at 120 V. Then, the gel photograph was taken from the gel visualization system.

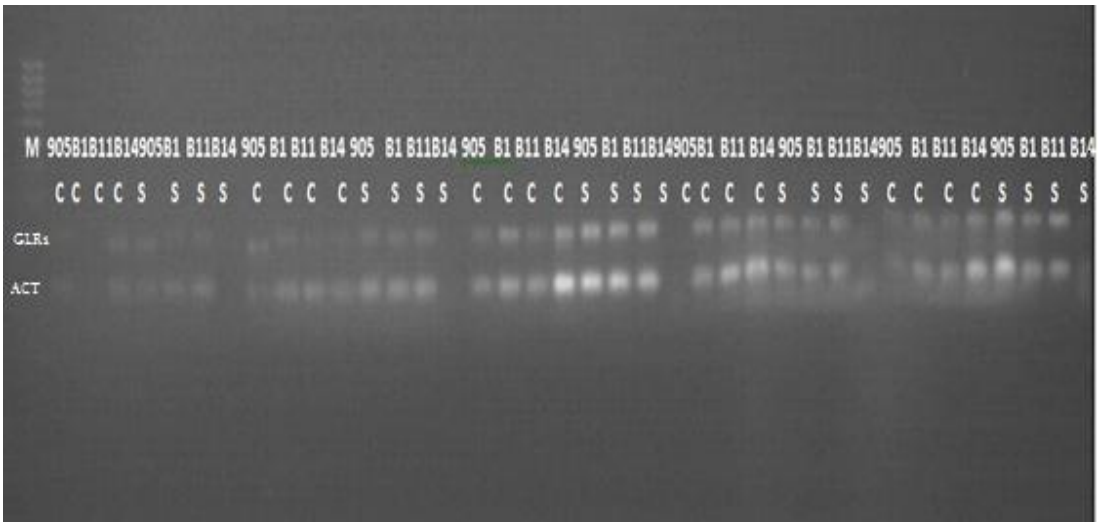


Figure 3.35 : RT-PCR experiment with GLR1 primer **Line 1** Fermentas Mass Ruler DNA Ladder Mix individuals and theirs expression profile is shown on the figure above for 24^{nd}, 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle respectively.

3.6.3.5 HSP104 cycle determination of selected individuals and wild type

The expression level of HSP104 was determined through RT-PCR by using ACT1 as internal control. RT-PCR application was done by using RNA samples of wild type and individuals which were isolated from cultures grown on YMM and 1 mM H_2O_2 containing YMM. According to their RNA concentrations, calculation was done and 100 ng RNA from each sample was used for RT-PCR studies. In order to determine at which cycle PCR product become saturated, PCR was lasted for 36 cycle and 10 µl sample was taken at 24nd, 27th, 30th, 33th, 36th cycle. These PCR products were loaded to agarose gel (1.5%).

Table 3.99 : RT-PCR cycle conditions for HSP104 gene

	Temperature (ºC)	Time	Cycle number
cDNA synthesis	55	30 min	1
Denaturation	94	2 min	1
PCR			
Denaturation	94	15 sec	**36**
Annealing	56	90 sec	
Extension	68	50 sec	
Final extension	68	5 min	1

PCR products taken at different PCR cycles were loaded to agarose gel and it was run for 30 min at 120 V. Then, the gel photograph was taken from the gel visualization system.

Figure 3.36 : RT-PCR experiment with HSP104 primer Line 1 Fermentas Mass Ruler DNA Ladder Mix ,individuals and theirs expression profile is shown on the figure above for 24^{nd}, 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle respectively.

3.6.3.6 YAP5 cycle determination of selected individuals and wild type

The expression level of YAP5 was determined through RT-PCR by using ACT1 as internal control. RT-PCR application was done by using RNA samples of wild type and individuals which were isolated from cultures grown on YMM and 1 mM H_2O_2 containing YMM. According to their RNA concentrations, calculation was done and 100 ng RNA from each sample was used for RT-PCR studies. In order to determine at which cycle PCR product become saturated, PCR was lasted for 36 cycle and 10 µl sample was taken at 24^{nd}, 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle. These PCR products were loaded to agarose gel (1.5%).

Table 3.2010 : RT-PCR cycle conditions for YAP5 gene.

	Temperature (ºC)	Time	Cycle number
cDNA synthesis	55	30 min	1
Denaturation	94	2 min	1
PCR			
Denaturation	94	15 sec	**36**
Annealing	53	90 sec	
Extension	68	50 sec	
Final extension	68	5 min	1

PCR products taken at different PCR cycles were loaded to agarose gel and it was run for 30 min at 120 V. Then, the gel photograph was taken from the gel visualization system.

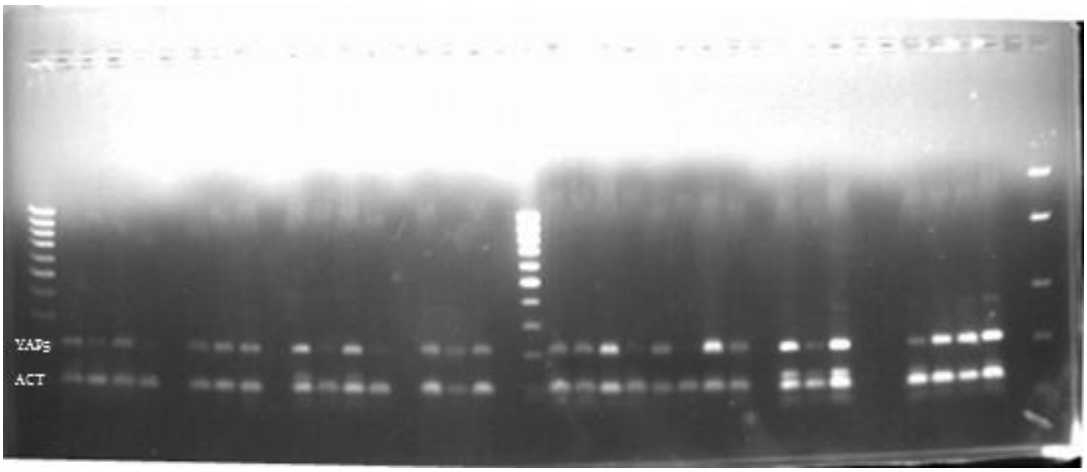


Figure 3.37 : RT-PCR experiment with YAP5 primer Line 1and 21: Fermentas Mass Ruler DNA Ladder Mix Line 40:, Fermentas Low Range DNA Ladder individuals and theirs expression profile is shown on the figure above for 27^{th}, 30^{th}, 33^{th}, 36^{th} cycle respectively.

3.6.4 Expression profile of relevant genes of wild type and selected individuals

3.6.4.1 CTT1 expression profile of wild type and individuals

Data from cycle determination was shown that PCR products were saturated at 36th cycle, so that cycle was accepted to visualize expression profile. The PCR products become saturated so, PCR was lasted at 36th cycle. The results of 36th cycle are shown on Figure 3.37.

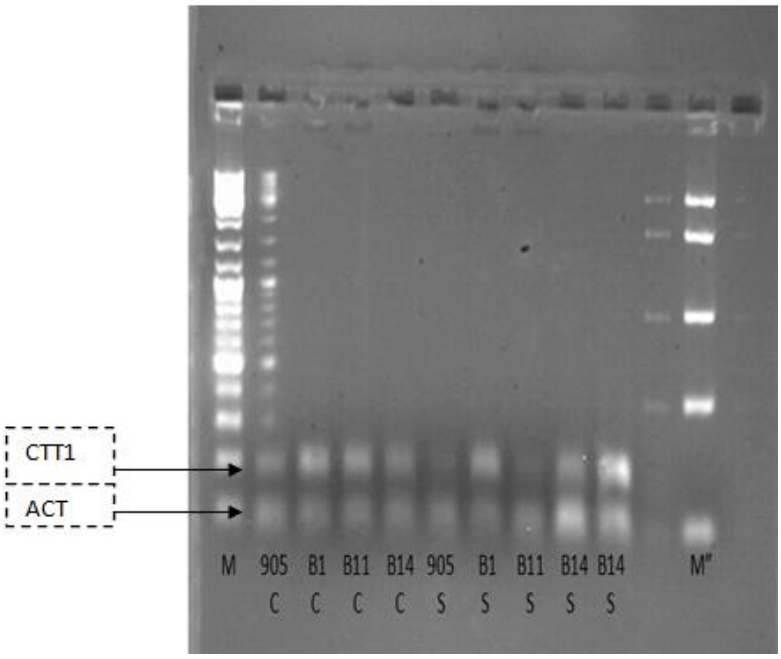


Figure 3.38 : Repeated RT-PCR experiment with CTT1 primer at 33th cycle. (1 – Fermentas DNA Ladder Mix (#SM0333), 12 - Fermentas DNA Low Range DNA Ladder (#SM1103) 3-905C, 4 – B1C, 5 – B11C, 6 – B14C, 7 -905S, 8 – B1S, 9 - B11S, 10 – B14S

The volume of the band was determined and calculated with UV-Spot Program and the results are shown on the Figure 3.38.

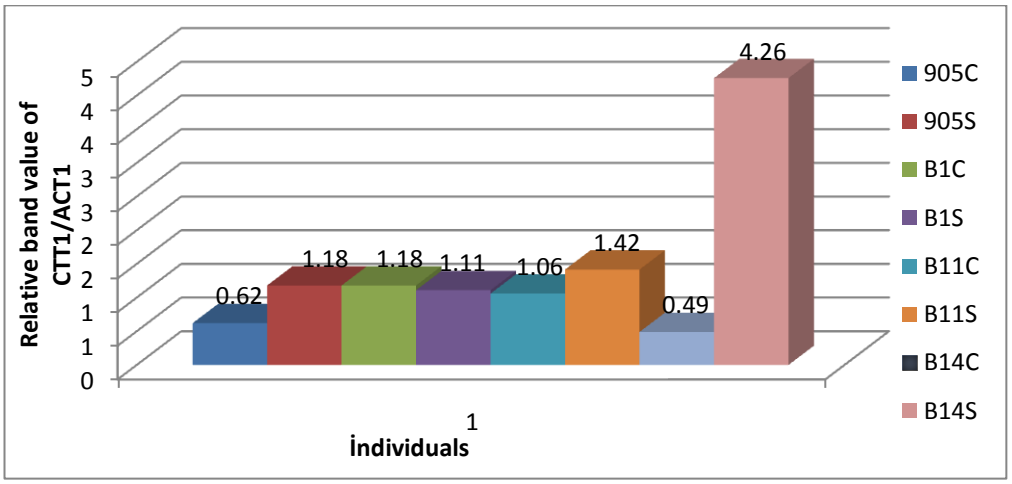


Figure 3.39 : Relative expression of CTT1

3.6.4.2 YAP1 expression profile of wild type and individuals

Data from cycle determination was shown that PCR products were saturated at 36th cycle, so that cycle was accepted to visualize expression profile. The PCR products become saturated so, PCR was lasted at 36th cycle. The results of 36th cycle are shown on Figure 3.40.

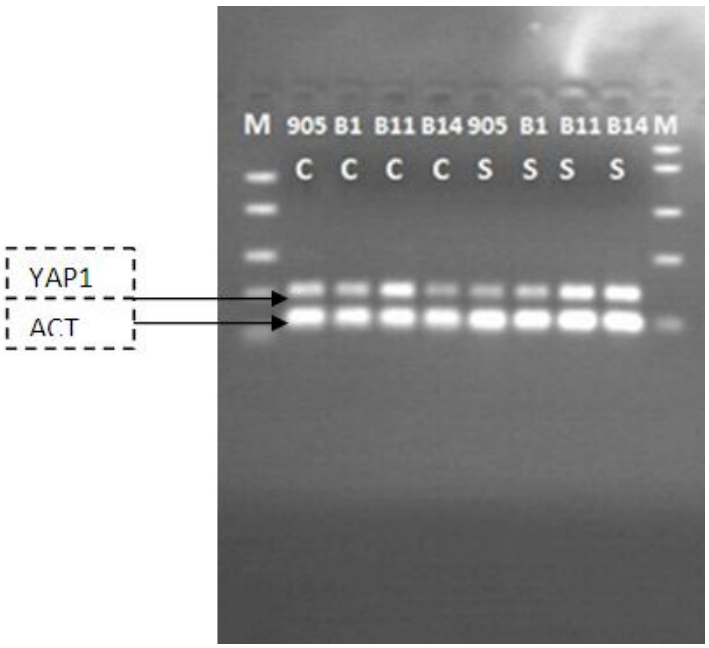


Figure 3.40 : Repeated RT-PCR experiment with YAP1 primer at 36th cycle.(1 and 10 Fermentas DNA Low Range DNA Ladder (#SM1103) 2 -905C, 3 – B1C, 4 – B11C, 5 – B14C 6 -905S, 7 – B1S, 8 - B11S, 9 – B14S

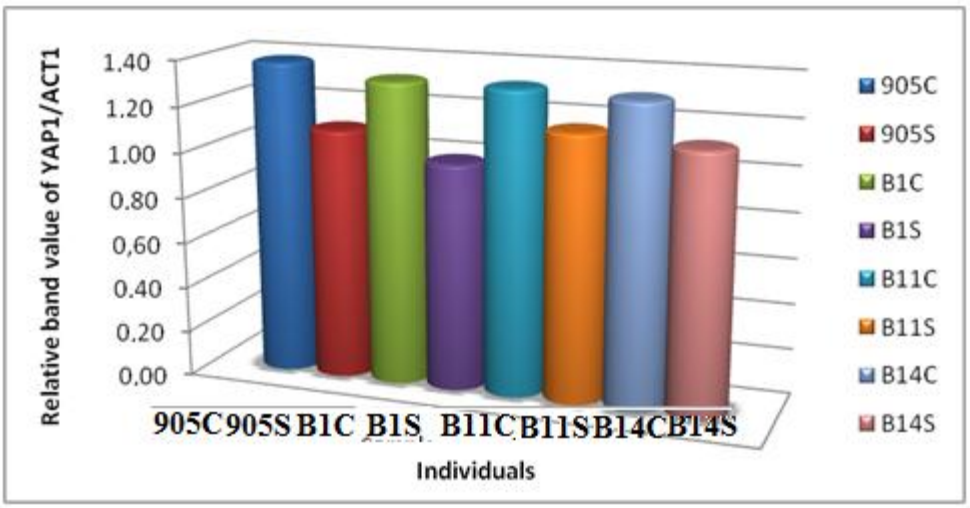


Figure 3.41 : Relative expression of YAP1.

3.6.4.3 ATF1 expression profile of wild type and individuals

Data from cycle determination was shown that PCR products were saturated at 36th cycle, so that cycle was accepted to visualize expression profile. The PCR products become saturated so, PCR was lasted at 36th cycle. The results of 36th cycle are shown on Figure 3.42.

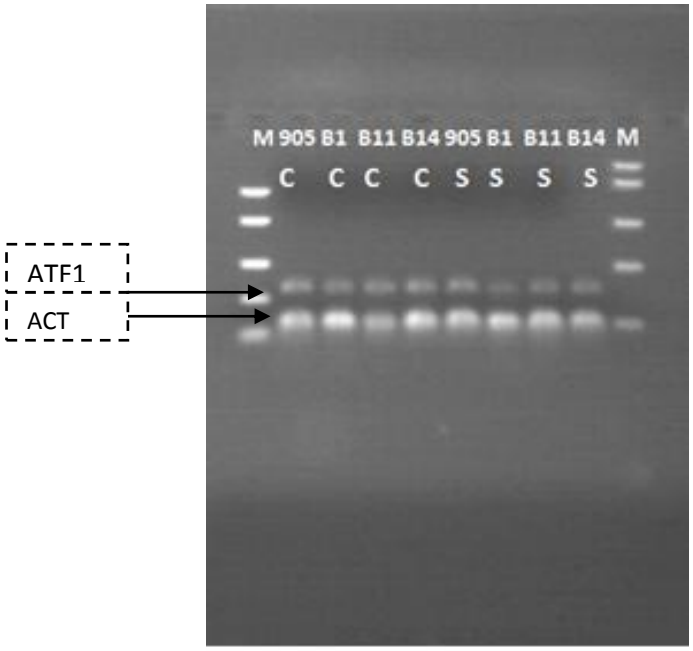


Figure 3.42 : Repeated RT-PCR experiment with ATF1 primer at 36th cycle.(1 and 10) Fermentas DNA Low Range DNA Ladder 2 -905C, 3 – B1C, 4 – B11C, 5 – B14C, 6 -905S, 7 – B1S, 8 - B11S, 9 – B14S

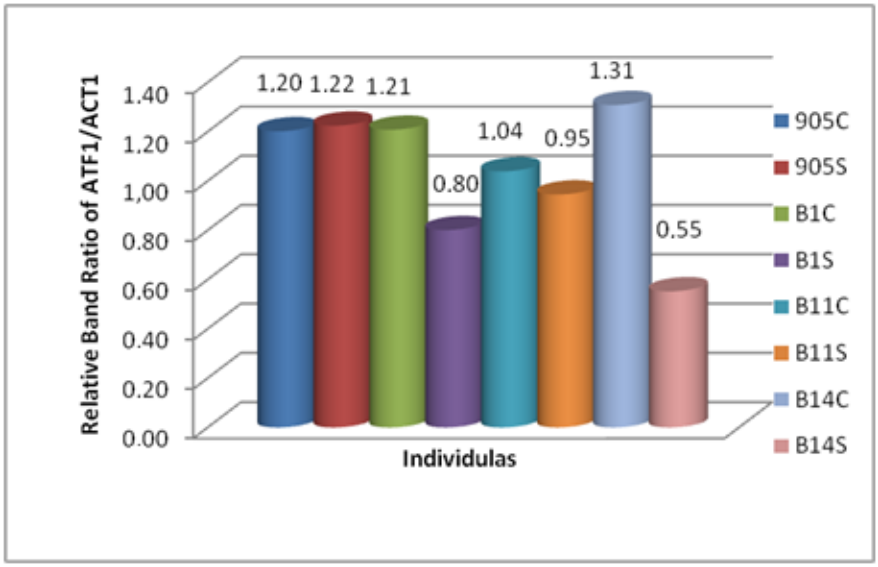


Figure 3.43 : Relative expression of ATF1

3.6.4.4 GLR1 expression profile of wild type and individuals

Data from cycle determination was shown that PCR products were saturated at 36th cycle, so that cycle was accepted to visualize expression profile. The PCR products become saturated so, PCR was lasted at 36th cycle. The results of 36th cycle are shown on Figure 3.44.

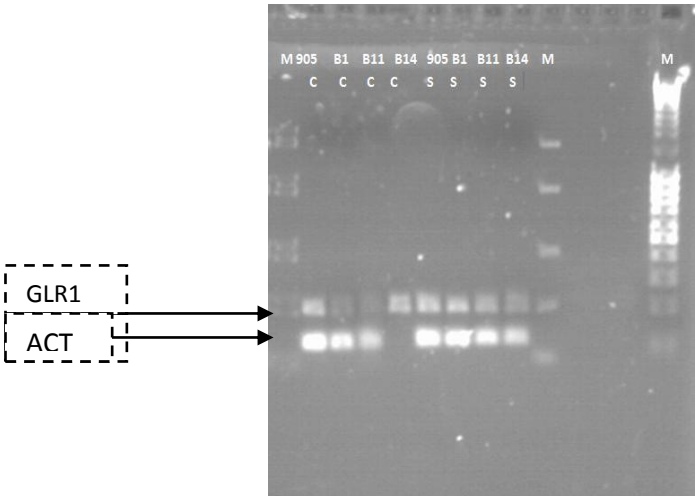


Figure 3.44 : Repeated RT-PCR experiment with GLR1 primer at 36th cycle.(1 and 10 Fermentas DNA Low Range DNA Ladder 2 -905C, 3 – B1C, 4 – B11C, 5 – B14C, 6 -905S, 7 – B1S, 8 - B11S, 9 – B14S

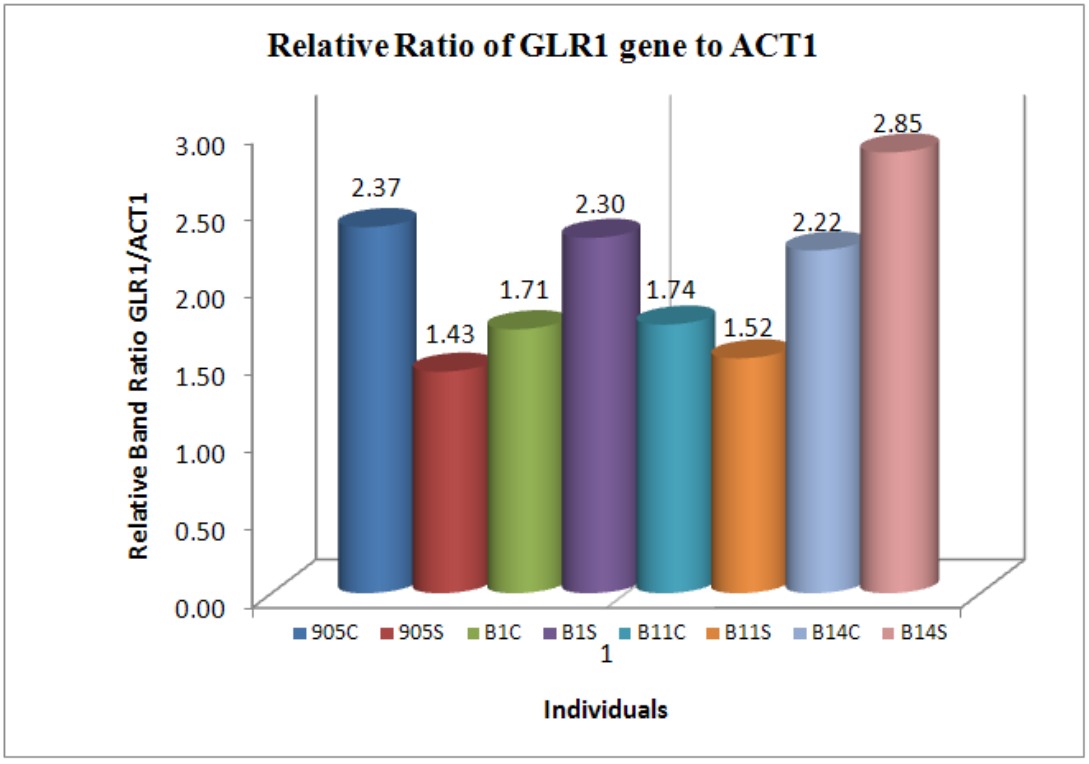


Figure 3.45 : Relative expression level of GLR1 to ACT1 for 336th PCR-cycle.

3.6.4.5 HSP104 expression profile of wild type and individuals

Data from cycle determination was shown that PCR products were saturated at 36th cycle, so that cycle was accepted to visualize expression profile. The PCR products become saturated so, PCR was lasted at 36th cycle. The results of 36th cycle are shown on Figure 3.46.

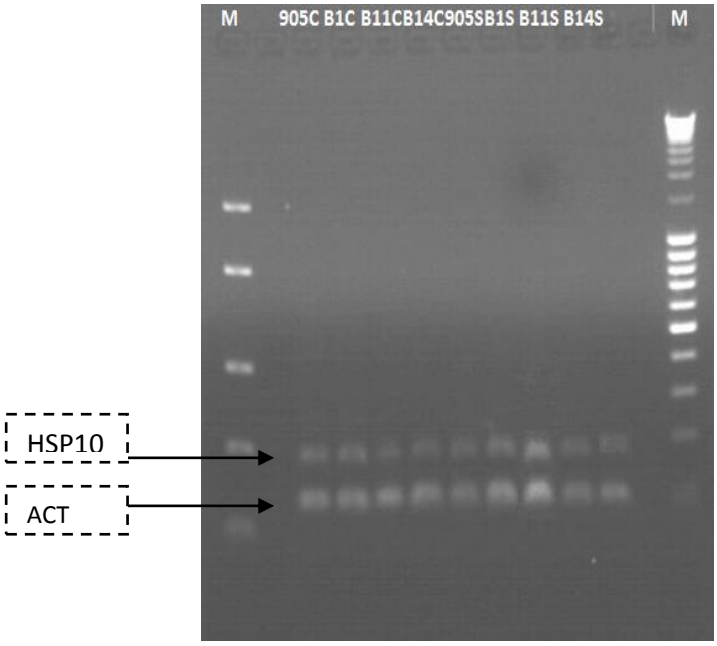


Figure 3.46 : Repeated RT-PCR experiment with HSP104 primer at 33th cycle. Line **1** Fermentas DNA Low Range DNA Ladder Line 2: Fermentas Mass Ruler DNA ladder **2** -905C, **3** – B1C, 4 **–** B11C**, 5** – B14C, **6** -905S, **7** – B1S, 8 - B11S**, 9** – B14S

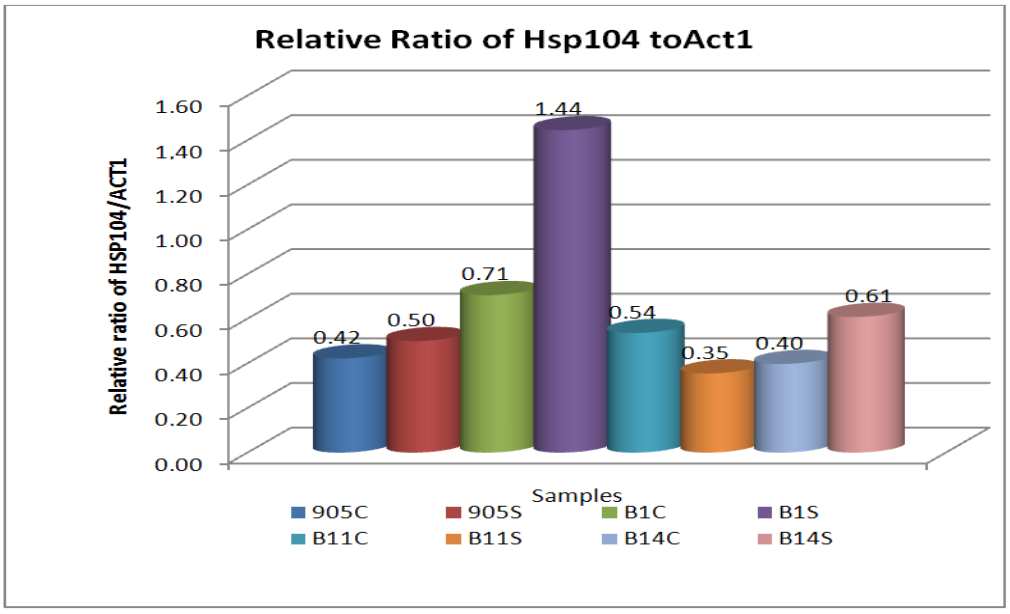


Figure 3. 47 : Relative expression level of HSP104 to ACT1 for 36th PCR-cycle.

3.6.4.6 YAP5 expression profile of wild type and individuals

Data from cycle determination was shown that PCR products were saturated at 36th cycle, so that cycle was accepted to visualize expression profile. The PCR products become saturated so PCR was lasted at 36th cycle. The results of 36th cycle are shown on Figure 3.48.

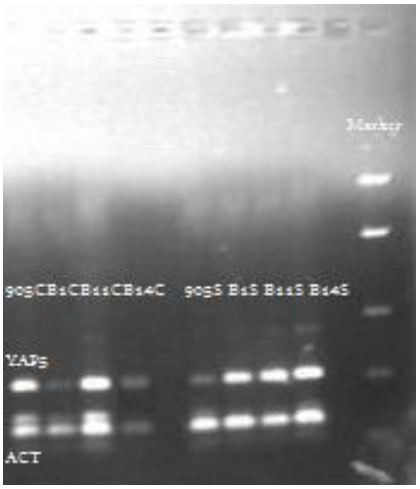


Figure 3.48 : Repeated RT-PCR experiment with YAP5 primer at 36th cycle.
1- 905C, **2** – B1C, **3 –** B11C**, 4** – B14C, **6** -905S, **7** – B1S,
8 - B11S**, 9** – B14S **10-** Fermentas DNA Low Range DNA Ladder (#SM1103)

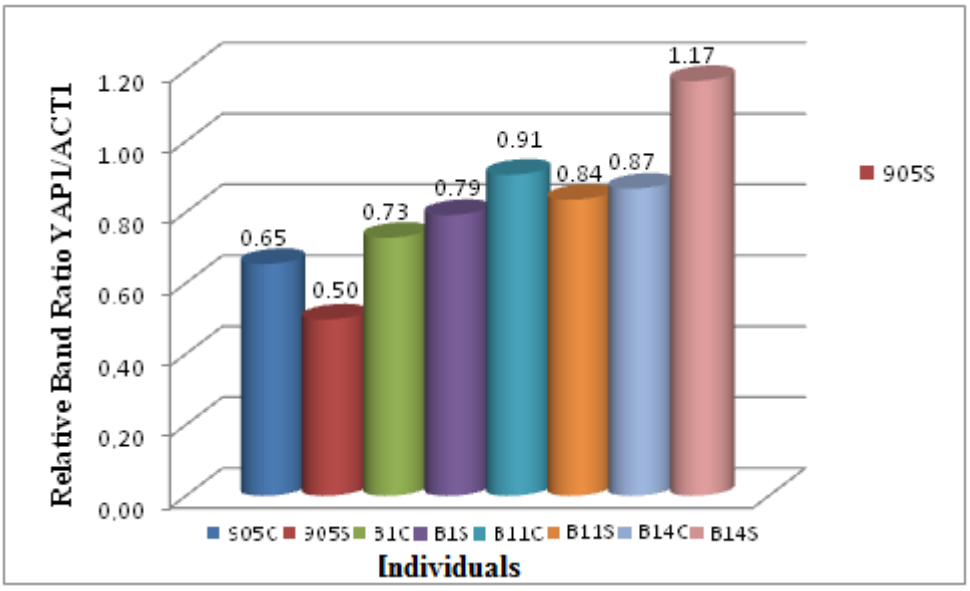


Figure 3.49 : Relative expression level of YAP5 to ACT1 for 36th PCR-cycle.

4. DISCUSSION and CONCLUSION

The ultimate goal of this study was to obtain hydrogen peroxide resistant *Saccharomyces cerevisiae* cells by using evolutionary engineering strategies. For that purpose, EMS was used as chemical mutagenesis, which causes many mutations through the genome and by this way, genetic variation was obtained.

The mutant population and wild type was exposed to different hydrogen peroxide stress levels to determine the highest stress level that the wild type and the mutant culture could still survive. Both cultures were shown to have tolerance 5 mM H_2O_2 thus the initial stress level was accepted as 5 mM H_2O_2.

Survivors of subsequent stages of stress selection were exposed to increasing levels f pulse stress and forty-six generations were obtained. The initial stress level was 5 mM H_2O_2 and that level was increased up to 50 mM H_2O_2 and 46^{th} generation was accepted as the final generation. After that step, individual mutants were selected randomly from the final populations. Thus final generation and individuals were tested for their hydrogen peroxide resistance by using 5-tube MPN strategy. The survival of the final population increased up to 11.371 and 1.989.967 fold of the wild type under continuous 0.1 and 0.2 mM H_2O_2 stress respectively. The survival of the final population increased up to 7809 fold of the wild type under pulse 0.1 H_2O_2 stress level.

The population and individuals were screened under other stress conditions such as heat, freeze-thaw, metal, ethanol and osmotic stress.

The results demonstrated that there was no growth observed for 905 and all individuals on YMM agar plates that contained 2 mM sorbitol (w/v); 6 % NaCl (v/v), 4 % NaCl (v/v), respectively for the 72^{nd} hour of incubation. It can be concluded that these results individuals could not gain cross resistance to osmotic stress. Thus, hydrogen peroxide resistance mechanism does not seem to be related to osmotic stress resistance mechanism.

Among the numerous environmental stresses, temperature change is one of the most common stress types for all living organisms. Heat shock and freeze thaw experiments were performed to investigate to relationship between oxidative stress and temperature shock. Heat shock proteins are serving as molecular chaperones, thus cells exposed to elevated temperature may have increased the synthesis of those proteins. Heat shock proteins are also key players that modulate the oxidative stress response as well as general stress response. The population and individuals were exposed to 60^0 C temperature stress for 10 min. B3 and B13 showed very little resistance to heat stress. The same situation was remained valid for cold shock and freeze-thaw stress. B14 was slightly resistant to both cold shock and freeze-thaw stress.

None of the individuals gained cross resistance to metals such as copper and chromium, but B11, B12 and B14 seemed to have gained cross-resistance toward iron stress. Negatively charged DNA binds transition metals cations, such as iron, thus iron metabolism and iron uptake might be important for oxidative stress (Hohmann and Mager, 2003). Iron metabolism and stress tolerance are also related with catalase activity. Degradation of hydrogen peroxide upon the catalase activity might be useful for adaptation to high cytoplasmic iron concentration, because of Fenton Reaction. The specific catalase activity of the B1, B11 and B14; and the CTT1, ATF1, YAP5 expression profile are compatible with each other.

The growth properties of wild type and individuals were investigated with and without hydrogen peroxide. The data shows that hydrogen peroxide treatment cause accumulation of toxic products, thus rapid entry to stationary phase and longer period of stationary phase were demonstrated. The interesting results were obtained from growth curve of wild type and B1, B11 and B14. The growth properties of these individuals were not different in the absence and presence of 1mM H_2O_2 continuous stress conditions, based on OD_{600} measurements. These results showed that the evolutionary engineering strategy is applied successfully and the individuals adopt the environment in perfect manner.

Catalase represents a family of detoxification enzymes that play an important role in resistance to toxic compounds in both prokaryotic and eukaryotic organisms. Specific catalase activity measurement of the wild type and selected individuals “B1, B11 and B14” was performed but only B14 showed higher activity in the absence

and presence of 1 mM H_2O_2 continuous stress conditions. On the other hand the data for B1 and B11 were not reliable, because catalase activity analysis was performed three times only B14 has shown the same result for each experiment.

Reverse-transcriptase PCR was performed at the end of the experiment and provide quantitative data about the expression profile associated with the populations and individuals comparatively. CTT1, YAP1, AFT1, GLR1, HSP104 and YAP5 are the genes that were investigated in this study, but more complex analyses such as microarray would provide better results.

Expression of the *S.cerevisiae* gene coding for a cytoplasmic catalase T (CTT1) and specific catalase activity was measured. CTT1 gene expression profile enhanced two times in stress conditions and the results seem to be consistent with literature data (Derek, et al., 2001). The specific catalase activity of wild type yeast cells also increased depends on hydrogen peroxide exposure time. The data that results from specific catalase activity and the gene expression profile were also consistent for the wild type.

The relative expression profile of CTT1 to ACT1 is higher than that of the wild type in non-stress conditions for B1 and B11; but in hydrogen peroxide stress conditions, B1 and B11 showed the same expression profile as the wild type. Although a higher expression level was observed, the specific catalase activity of B1 has not higher than the wild type in non-stress conditions. This may be related to regulation of gene translation, because the expression occurs at the same level in stress and non-stress conditions, so the translation is repressed until higher hydrogen peroxide levels. The situation is opposite for B11, because it shows both higher specific catalase activity and CTT1 expression.

The relative expression profile of CTT1 also showed that B14 had the highest catalase activity. It was clearly seen that B14 had 8-fold more expression level than the wild type and that result correlated with specific catalase activity result. The interesting result is in non-stress condition, B14 has higher expression profile than the wild type and individuals “B1 and B11” which was exposed to hydrogen peroxide stress. The mutation might have caused to alter the specificity of RNA polymerase for B14’s CTT1 gene and making it more likely to bind to CTT1 gene, or

enhance the interaction between RNA polymerase and a CTT1 promoter by a mutant protein that shows enhancer property.

Yap1 is a transcription factor and activates expression of antioxidant genes in response to oxidative stress, thus under oxidative stress conditions, Yap1 expression level is expected to increase but related to regulation of other genes depends on time, the expression level decrease. YAP1 also regulates GLR1 expression. The YAP1 expression profile for wild type and selected individuals is not different in the stress and non-stress conditions. The EMS mutagenesis did not seem to affect the expression profile or structural design of YAP1. The exposure of hydrogen peroxide causes the induction of YAP1 expression and that leads to regulate expression of other genes, but at the time point that the samples were taken, the hydrogen peroxide response had already been initiated. The redox-dependent Yap1 localization might also affect YAP1 expression and Yap1p expression may be down regulated for wild type and selected individuals.

Glr1 is an antioxidant gene that is responsible for maintaining the necessary level of GSH. YAP1 regulates the expression of GLR1 in precedence of oxidants. The GSSG to GSH ratio regulates the oxidative stress response, thus the specific ratio needs to be conserved. The GLR1 gene expresses Glutathione reductase enzyme which conserves GSSG to GSH ratio with the utilization of glutathione by NADPH. The expression profiles of B1 and B14 showed that high intracellular GSSG to GSH ratio might be reduced to its oxidized glutathione form by NADPH-dependent manner. This result is also indicated in the literature. (Grant, et al., 1998)

On the other hand, the oxidized form (GSSG) could be degraded or the other enzymes like transhydrogenase might effective to convert its reductive form (GSH) to get rid of its toxic effect that inhibit protein synthesis (Grant, 2001). However, all individuals have higher GLR1 expression levels than the wild type, it can be generally concluded that the GLR1 activity increases in oxidative stress condition. The relation between Glr1 and Yap1 could not be identified using the experimental data. Additionally, Skn7p might also be responsible for oxidative stress response.

Heat shock protein expression is highly sensitive to oxidative stress, but heat shock proteins are widely separated. Heat shock proteins work as molecular chaperons in

general stress mechanism. In a literature study, upon exposure to 0.4 mM H_2O_2, yeast cells HSP104 expression was induced 14.9 times (Godon, et al., 1998). In that study, Hsp104 expression profile was slightly increased for wild type and B14 in the presence of hydrogen peroxide. In contrast, for B1, HSP104 expression slightly decreased in the presence of hydrogen peroxide. B1 shows three times higher HSP104 expression in the presence of hydrogen peroxide, but it did not affect its cross resistance to cold (-20^{o}C), temperature (60^{o}C) or freeze-thaw (-196^{o}C) stresses. The individual known as B14 had a survival ratio as fold of wild type 10,373 times upon 50 mM H_2O_2 pulse stress; and 8,696 times upon 0.1 M H_2O_2 pulse stress; 3,695,652 times upon 0.2 M H_2O_2 pulse stress. The experimental results of the study are consistent with the literature (Godon, et al., 1998).

The expression levels of YAP5 and ATF1 are discussed together. Yap5 is a transcription factor that regulates a set of genes involved in iron metabolism. Yap5 contains two cysteine rich domains and high iron levels in cytoplasm modify the sulfhydryls, and change the Yap5 conformation. The conformational changes of Yap5p cause constitutively occupying the CCC1 promoter and so the Ccc1p gets higher in the cytoplasm. Ccc1p is responsible for the transport of iron and manganese from cytosol to vacuole. The Atf1p is a transcriptional factor which regulates a set of genes involved in iron uptake and homeostasis. As cytoplasmic iron levels decrease, Atf1 is activated and induces Cth2 expression which binds and destabilizes Ccc1 mRNA, thus iron starvation and cobalt stress induce Aft1 accumulation in the nucleus. By that way, cytosolic iron level becomes higher because it lowers iron import into the vacuole. There was no growth obtained for wild type and individuals on YMM plate with 2 mM $CoCI_2$; stress at 72. hour of incubation but B11, B12 and B14 seemed to have gained co-resistance toward iron stress. According to those results, Yap5p and Aft1 were chosen as reference genes. The wild type has higher ATF1 expression levels in stress and non-stress conditions, but lower Yap5 activity than the mutant individuals. The B1, B11 and B14 have iron resistance 1.5 times, 21.88 times and 11.41 times than the wild type, respectively. And all of the mutant individuals have higher Yap5 expression and lower Atf1 expression. The results also showed that the Yap5 and Atf1 seem to have a reverse relationship. The B1 and B11 have lower specific catalase activity than B14. B14 has 8 time's higher CTT1 expression and also specific catalase activity, so the Fenton reaction seems to be

more active. The Fenton reaction causes Fe^{+3} accumulation on the cytoplasm and that causes the degradation of the lipid hydro peroxide (LOOH) to more reactive forms. Thus, the oxidative stress conditions show the importance of cytoplasmic iron transport to vacuoles. B14 has two fold more YAP5 expression profile than the wild type and in the same ratio lower ATF1 activity. That activity changes possibly help B14 to rapidly transport cytoplasmic iron to vacuoles and prevent the cell from toxic effects of iron.

B11 has showed slight increase in expression level of YAP5 in the presence of hydrogen peroxide, and also without hydrogen peroxide exposure the expression profile for YAP5 was higher than the wild type cells. Related to YAP5 activity, ATF1 expression profile is also below the wild type expression profile as expected. B11 has higher specific catalase activity and CTT1 expression, thus increases in cytosolic iron might have caused Yap5p activation like for B14.

Interestingly, B1 has the same expression profile like wild type for CTT1 and ATF1 without hydrogen peroxide exposure and specific catalase activity is not very different from the wild type in that condition. However, in the presence of hydrogen peroxide, YAP5 expression profile is higher and ATF1 expression profile is lower than the wild type, as expected. The catalase activity is also higher for the first 18 h, but then the same as wild type cells. The lower iron cross-resistance might be obtained because of that reason. The B1 cells only show high expression in the presence of stress conditions.

Cobalt resistance correlates with an increase in intracellular iron. (Stadler et. al., 2002). On the other hand, why iron resistant yeast cells could not obtain cobalt resistance is still not clarified. Thus, there might be another gene that regulates cobalt uptake and metabolism.

This study might be completed with screening of all genes involved in hydrogen peroxide detoxification, for that purpose the transcriptional profile of *Saccharomyces cerevisiae* upon hydrogen peroxide exposure have to be determined. RT-PCR experiments are only a limited part of transcription analyses. Further microarray analyses could provide us more detailed results. On the other hand, real time PCR experiments might provide the reliable quantitative data for understanding the genetic expression profiles in detail.

REFERENCES

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002: Molecular Biology of the Cell, 4th edition, Garland Science Publications, New York.

Álvarez, S., Rüfenacht, K., Eggen, R., 2002: The oxidative stress sensitive *yap1* null strain of *Saccharomyces cerevisiae* becomes resistant due to increased carotenoid levels upon the introduction of the *Chlamydomonas reinhardtii* cDNA, coding for the 60S ribosomal protein L10a, *Biochemical and Biophysical Research Communications*, **267**, 953-959.

Azevedo, D., Tacnet, F., Delaunay, A., Pousada, C., Toledano, M., 2003: Two redox centers within *Yap1* for H_2O_2 and thiol-reactive chemicals signaling, *Free Radical Biology and Medicine*, **35**, 889-900.

Baĭliak, M., Semchyshyn, M., Lushchak H., 2006: Role of catalase and superoxide dismutase in the yeast *Saccharomyces cerevisiae* response to hydrogen peroxide in exponential phase of growth, *Ukrainskii Biolhimicheskii Zhurnal,* **78,** 79-85.

Bayliak, M., Semchyshyn, H., Lushchak, V., 2007: Possible accumulation of non-active molecules of catalase and superoxide dismutase in *S. cerevisiae* cells under hydrogen peroxide induced stress, *Central European Journal of Biology*, **2**, 326–336.

Black G., J., 2002: Microbiology, Principles and explorations, 5th Edition, John Wiley & Sons, Inc., Nev Jersey

Betsy, T., Keogh, J., 2005: Microbiology Demystified, The McGraw-Hill Companies, Inc, New York.

Bitton ., 2005: Wastewater Microbiology, 3rd edition, John Wiley & Sons, Inc., Nev Jersey

Cakar, Z.P., Seker, U.O.S., Tamerler, C., Sonderegger, M. and Sauer, U., 2005: Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae, FEMS Yeast Research*, **5,** 569-578.

Carlile M. J., Watkinson S.C., Gooday G. W., 2001: The Fungi, Academic Press, London

Catala A., 2009: Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions , *Chemistry and Physics of Lipids* 157, 1–11

Costa V., Ferreira P. 2001: Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases, *Molecular Aspects of Medicine*, **22,** 217-246.

Costa V., Quintanilha A., Moradas-Ferreira P. 2007: Protein Oxidation, Repair Mechanisms and Proteolysis in *Saccharomyces cerevisiae*, *The IUBMB Life*, **59**, 293 – 298

Delanuay a., Isnard A., Toledano M. 2000: H_2O_2 sensing through oxidation of the Yap1 transcription factor, *The EMBO Journal*, **19,** 5157-5166.

Demasi, A. P., G. A. Pereira, and L. E. Netto. 2006: Yeast oxidative stress response. *FEBS Journal*. **273,** 805-816

Dounce A., 2004: A proposed mechanism for the catalatic action of catalase, *Journal of Theoretical Biology*, **105**, 553-567.

Derek J., 1998: Oxidative stress responses of the yeast *Saccharomyces cerevisiae*, *Yeast*, **14**, 1511 – 1527.

Drabkova, M., 2005: Study of Physicochemical and antioxidative properties of yeast membrane components, Phd. Thesis, Brno University of Technology, Purkynova , Czech Republic

Godon C., Lagniel G., Lee J., Buhler J., Kieffer S., Perrot M., Boucherie H., Toledano M., Labarre J., 1998: The H_2O_2 Stimulon in *Saccharomyces cerevisiae*, *The Journal of Biological Chemistry*, **273**, 22480-22489.

Govinden, R., B. Pillay, W.H. Van Zyl, and D. Pillay., 2001: Xylitol production by recombinant Saccharomyces cerevisiae expressing the Pichia stipitis and Candida shehatae XYL1 genes. *Applied Microbiology and Biotechnology* **55:** 76 - 80.

Grant, C., M., Perrone, G., Dawes, I., W., 1998. Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast Saccharomyces cerevisiae, *Biochemical and Biophysical Research Communications*, **253,** 893–898.

Grant, C., M., 2001. Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. *Molecular Microbiology*, **39**, 533-41.

Gulshan *K.*, *Rovinsky S.*, *Coleman S.*, *Rowley W,*. *2005:* Yeast oxidative stress response. Influences of cytosolic thioredoxin peroxidase I and of the mitochondrial functional state, *FEBS J.*, **273,** 805-16.

Gutteridge M., 1998: Lipid peroxidation: some problems and concepts, *Oxygen Radicals and Tissue Injury*, pp. 9–19.

Hogg S., 2005: Essential Microbiology, John Wiley & Sons Inc. Publications, Nev Jersey

Hohmann S., Mager W. H., 2003: Yeast stress response, Springer-Verlag Berlin Heidelberg, New York

Holmgren A., 1989: Thioredoxin and Glutaredoxin Systems, *The American Society for Biochemistry and Molecular Biology*, **264,** 13963-1396.

Holmgren A., Johansson C., Berndt C., LÖ M., Hudemann C., Lillig C., 2005: *Cellular Information Processing*, **33**, 1375- 1377.

Kayser 0., Muller H. R., 2004. Pharmaceutical Biotechnology, Drug Discovery and Clinical Applications, Wiley-VCH Verlag GmbH & Co, Weinheim.

Keeton, W.., Gould, J.L., 1999: Biological Science, 5th edition, Ankara, Palme Publicition.

Kuge S., Jones N., Nomoto A. 1997: Regulation of *yAP-1* nuclear localization in response to oxidative stress, *The EMBO Journal*, **16,** 1710–1720

Kuznetsov V., Radyukina N., Shevyakova N., 2006. Polyamines and Stress: Biological Role, Metabolism, and Regulation, *Russian Journal of Plant Physiology*, ***53,*** *583–604.*

Lawrence C.W., 1991: Classical mutagenesis techniques, *Methods Enzymology* **194,** 456-464

Lee J., Godon C., Lagniel G., Spector D., Garin J., Labarre J., Toledano M. 1999: Yap1 and Skn7 Control Two Specialized Oxidative Stress Response Regulons in Yeast, *The Journal of Biological Chemistry*, **274,** 16040–16046

Leister, D., Hermann, J, M., 2007 : Mitochondria: Practical Protocols, Humana Press Inc., USA

Lodish, H., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., Darnell, J., 1999 : Molecular Cell Biology, 4th edition, W.H. Freeman & Co., New York.

Mai B., Breeden L. 1997 : Xbp1, a stress-induced transcriptional repressor of the *Saccharomyces cerevisiae* Swi4/Mbp1 family. *Molecular Cell. Biology,* **11**, 6491-6501.

Mathews C., Van Holde K., 1990: Biochemistry, Benjamin-Cummings Publishing

Martinez C., Martins G., Bianchini M. 2009 : Metallothionein-like proteins in the blue crab *Callinectes sapi dus:* Effect of water salinity and ions, *Comparative Biochemistry and Physiology*, **152** 366–371

Moat A. G., Foster J. W., Spector M. P., 2002 : Microbial Physiology, Wiley-Liss, Inc., 4th edition, New York.

Molin M., Renault J., Lagniel G., Pin S., Toledano M., Labarre J. 2007: Ionizing radiation, induces a Yap1-dependent peroxide stress response in yeast *Free Radical Biology and Medicine*, **43**, 136-144.

Moore, M. S., 1998: Ran and Nuclear Transport, *The Journal of Biological Chemistry,* **273,** 22857–22860

Mustacich D., Powis G. 2000: Thioredoxin reductase, *Biochemistry*, ***346***, 1-8

Nguyen, H.T.T., Dieterich, A., Athenstaedt, K., Truong, N.H., Stahl, U.,Nevoigta E., 2004: Engineering of *Saccharomyces cerevisiae* for the production of l-glycerol 3-phosphate, *Metabolic Engineering*, **6,** 155–163

Postlethwait, J. H. and Hopson, J. L., 2006. Modern Biology, 1st Edition, Holt, Rinehart, and Winston, Anzahl der Seiten

Rehm, H.J., Reed, G., Puhler, A., Stadler, P., Stephanopoulos G., 1991: Biotechnology, 2nd Edition Volume 3/Bioprocessing

Roehr M., 2001: The Biotechnology of Ethanol: Classical and Future Applications, Wiley-VCH Verlag GmbH & Co, Weinheim.

Ross S., Findlay V., Malakasi P., Morgan B. 2000: Thioredoxin Peroxidase is required for the transcriptional response to oxidative stress in budding yeast, *Molecular Biology of the Cell,* **11**, 2631–2642.

Rowley, M., Harshman, K. D., Parker, C.S., *1998:* Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins, *Genes and Development,* **3,** 283-292

Scandalios J. 1993: Oxygen Stress and Superoxide Dismutases, *Plant Physiology,* **101,** 7-12

Scheffler I. E., 2008: Mitochondria, 2nd edition, John Wiley & Sons Inc. Publications, Nev Jersey

Schrader M., Fahimi H. 2006: Peroxisomes and oxidative stress, *Biochimica et Biophysica Acta* **1763,** 1755–1766

Skory, C. 2003: Lactic acid production by *Saccharomyces cerevisiae* expressing a *Rhizopus oryzae* lactate dehydrogenase gene. *Journal Indian Microbiology and Biotechnoogy,* **30:**22-27

Smits, G., J., Brul, S., 2005: Stress tolerance in fungi to kill a spoilage yeast, *Current Opinion Biotechnology* 16, 225 – 228

Sugiyama K., Izawa S., Inoue Y. 2000. The Yap1p-dependent Induction of glutathione Synthesis in Heat Shock Response of *Saccharomyces cerevisiae The Journal of Biological Chemistry*, 275(20), 15535–5540.

Url-1 < http://www.gmm.gu.se/groups/hohmann/uploads/yeast.pdf >, accessed at 22.06.2009.

Turanlı B., 2006: Evolutionary Engineering of Metal-binding / Metal-resistant Yeast for Biomimetics, Msc. Thesis, Istanbul Technical University, İstanbul, Turkey

Tkachenko A.; Nesterova L., Pshenichnov P. 2001: The role of the natural polyamine putrescine in defense against oxidative stress in *Escherichia coli Arch Microbioogy,* **176,** 155–157

APPENDIX A

BUFFERS

TBE (Tris-Borate-EDTA) Buffer (10X)

Tris base	108 g
Boric Acid	55 g
EDTA	40 ml (0.5 M, pH 8.0)

Add ddH_2O to 1 liter and adjust the pH to 8.0

Mini Agarose Gel (1.5 %)

Agarose	0.75 g
TBE buffer (1X)	50 mL

Add 2.5 µL EtBr before pouring the gel into tray.

Midi Agarose Gel (1.5 %)

Agarose	2.25 g
TBE buffer (1X)	150 mL

Add 7.5 µL EtBr before pouring the gel into tray.

10X Phosphate Buffered Saline (PBS)

NaCl	90 g
$Na_2HPO_4.7H_2O$	26.8 g
NaH_2PO_4	3.2 g

Add up to 1 L by ddH_2O, and adjust the pH to 7.2 after every 1/10 dilution.

APPENDIX B: MPN Index For 5 Test Tubes

MPN Index for Combinations of Positive and Negative Results When Five Tubes Are Used per Dilution (five each of 10 ml, 1 ml, and 0.1 ml)

Number of Tubes with Positive Results				Number of Tubes with Positive Results			
10 ml	1 ml	0.1 ml	MPN Index/100 ml	10 ml	1 ml	0.1 ml	MPN Index/10(
0	0	0	<2	4	3	1	33
0	0	1	2	4	4	0	34
0	1	0	2	5	0	0	23
0	2	0	4	5	0	1	30
1	0	0	2	5	0	2	40
1	0	1	4	5	1	0	30
1	1	0	4	5	1	1	50
1	1	1	6	5	1	2	60
1	2	0	6	5	2	0	50
2	0	0	4	5	2	1	70
2	0	1	7	5	2	2	90
2	1	0	7	5	3	0	80
2	1	1	9	5	3	1	110
2	2	0	9	5	3	2	140
2	3	0	12	5	3	3	170
3	0	0	8	5	4	0	130
3	0	1	11	5	4	1	170
3	1	0	11	5	4	2	220
3	1	1	14	5	4	3	280
3	2	0	14	5	4	4	350
3	2	1	17	5	5	0	240
4	0	0	13	5	5	1	300
4	0	1	17	5	5	2	500
4	1	0	17	5	5	3	900
4	1	1	21	5	5	4	1600
4	1	2	26	5	5	5	≥1600
4	2	0	22				
4	2	1	26				
4	3	0	27				

(Black, 2002)

CURRICULUM VITAE

Candidate's full name: Meliha Merve HIZ

Place and date of birth: Samsun, 25.06.1985

Permanent Address : Barbaros Mah. Özdil Sokak İdealkent Sitesi G Blok D:3 Çanakkale/Türkiye

Universities and Colleges attended: B.Sc.**;** Istanbul University, Science Faculty, Department of Molecular Biology and Genetics, 2007.